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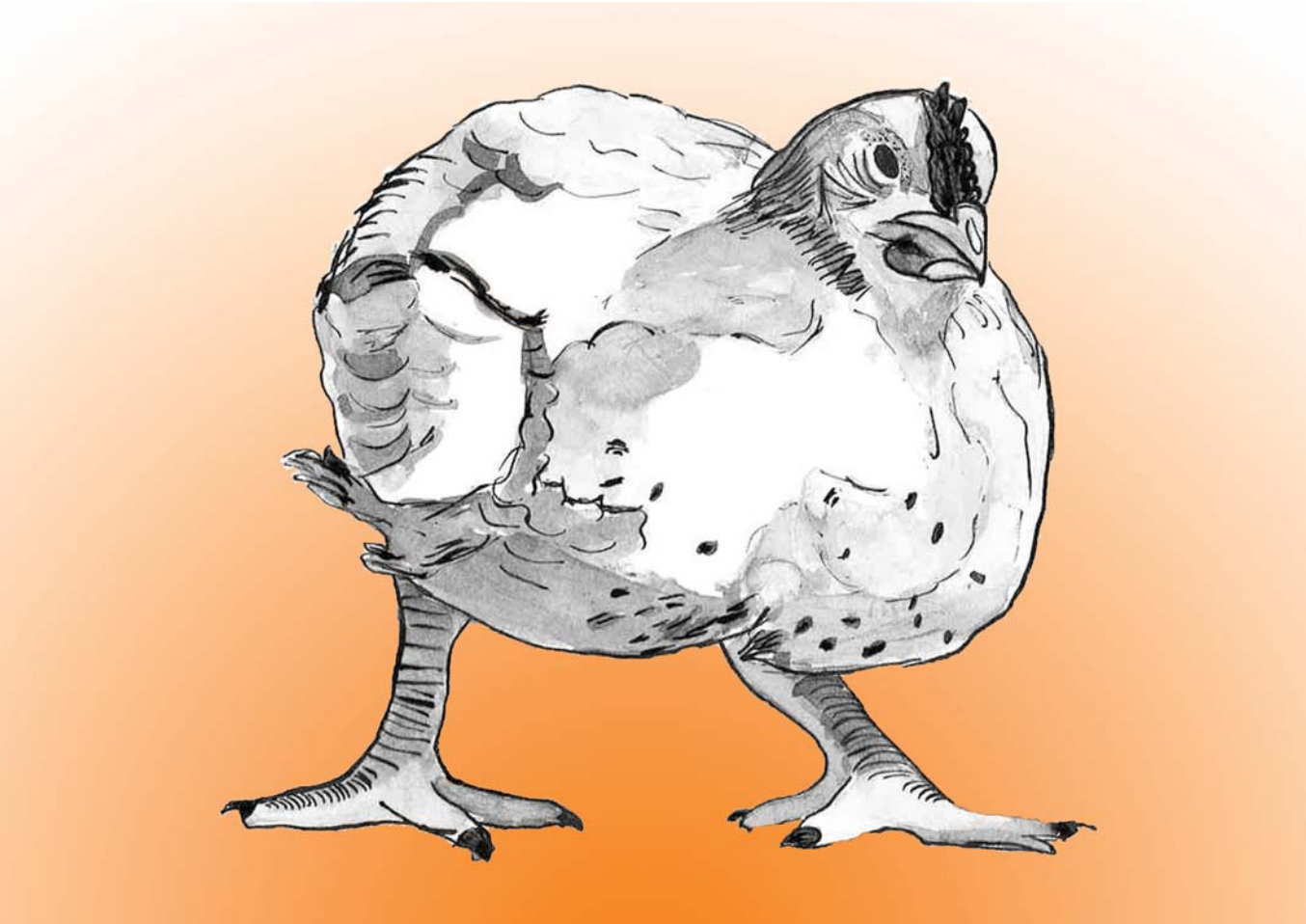
SURVIVAL AND REDUCTION OF STRAINS OF *CAMPYLOBACTER* SPECIES IN BROILER MEAT

PAULIINA ISOHANNI



SURVIVAL AND REDUCTION OF STRAINS OF *CAMPYLOBACTER* SPECIES IN BROILER MEAT

PAULIINA ISOHANNI

Academic Dissertation in Biotechnology
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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. Katzav M., Isohanni P., Lund M., Hakkinen M. & Lyhs U. 2008, "PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products", *Food Microbiology,* vol. 25, pp. 908-914.

II. Isohanni, P.M.I. & Lyhs U. 2009, "Use of ultraviolet irradiation to reduce *Campylobacter jejuni* on broiler meat", *Poultry Science,* vol. 88, pp. 661-668.

III. Isohanni P., Alter T., Saris P., & Lyhs, U. 2010, "Wines as possible meat marinade ingredients possess antimicrobial potential against *Campylobacter*", *Poultry Science,* vol. 89, pp. 2704-2710.

IV. Isohanni P., Huehn S., Aho T., Alter T., and Lyhs U. 2013, "Heat stress adaptation induces cross-protection against lethal acid stress conditions in *Arcobacter butzleri* but not in *Campylobacter jejuni*". *Food Microbiology,* vol. 34, pp. 431-435.

The publications are indicated in the text by their Roman numerals. The original articles have been reprinted with the permission of their copyright holders.

ABBREVIATIONS

ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BIOHAZ	EFSA Panel on Biological Hazards
BPW	Buffered Peptone Water
BSA	Bovine Serum Albumin
CASO	Casein-Peptone Soymeal-Peptone Agar
cfu	colony forming units
CMJ	Chicken Meat Juice
DNA	deoxyribonucleic acid
D-value	decimal reduction time
EFSA	European Food Safety Authority
ICMSF	International Commission on Microbiological Specifications for Foods
J/cm^2	joules per square centimetre
mCCDA	Modified Charcoal Cefoperazone Deoxycholate Agar
MHSB	Mueller-Hinton agar with Sheep Blood
mWs/cm^2	milliwatts per second per square centimetre
NCFA	Nordic Committee of Food Analyses
NCTC	National Collection of Type Cultures
OD_{600}	optical density at 600 nm
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
THL	National Institute for Health and Welfare
TSA	Trypticase Soy Agar
UV	ultraviolet
VBNC	viable but non-cultivable
WHO	World Health Organization

ABSTRACT

Campylobacter, especially *Campylobacter jejuni* and *C. coli*, are reported as the most frequent cause of foodborne bacterial diarrhoea in humans worldwide. One of the most important sources of human campylobacteriosis is the eating or handling of improperly cooked or raw broiler meat.

Between January and September 2006, 194 marinated and non-marinated poultry (broiler and turkey) meat products were collected from retail shops in Western Finland and tested for *Campylobacter* using conventional enrichment culture and Polymerase Chain Reaction (PCR) methods. For marinated poultry products, the study involved modification of a commercial DNA isolation method. Using either method, a total of 25 (12.9%) of all the samples were *Campylobacter* positive. In August, there was a peak with 28.9% positive samples. In marinated poultry products, *Campylobacter* was detected at a prevalence of 10.3%. Due to the high detection limit of the direct *Campylobacter* PCR method, it was necessary to perform a combination of enrichment and PCR.

The effects of ultraviolet (UV) irradiation in reducing *C. jejuni* E1 1347 on the surfaces of broiler meat, skin and carcasses were studied. The surfaces were inoculated with varying counts of *C. jejuni* E1 1347 and treated with UV irradiation with doses ranging between 9.4 and 32.9 milliwatts per second per square centimetre (mWs/cm^2). The log reductions in *C. jejuni* E1 1347 counts were determined by dilution plating. The effects of UV irradiation on the sensory quality of broiler meat were also evaluated. The maximum reduction achieved was 0.7 log on broiler meat, 0.8 log on broiler skin and 0.5 log on carcasses. UV irradiation did not affect the sensory quality of broiler meat.

The survival of high (7 log cfu/ml) and low (3 log cfu/ml) inocula levels of *Campylobacter* strains RefCJ, RetCJ29, RetCC27 and SlaCJ26 was studied in white and red wines, and in grape and tomato juices. For comparison, survival was studied in a commercial poultry meat marinade. The log reductions were determined by dilution plating. High counts of the bacteria were rapidly inactivated to undetectable numbers within 15 min in white wine and within 1 h in red wine, and low counts within 15 min in white wine and within 30 min in red wine. In grape and tomato juices even low counts of the bacteria were occasionally detected after 48 h. In the commercial marinade the high bacterial counts were inactivated in most cases within 48 h and all the low counts within 3 h. When *Campylobacter* strains RefCJ and RetCC27 were inoculated on broiler meat, wines reduced the bacterial counts by approximately 1 log cfu/ml over 48 h.

Arcobacter are close phylogenetic relatives of *Campylobacter* that have occurred in broiler meat and have been linked with human illness (mainly diarrhoea). Sublethal stress adaptation temperatures (48°C and 10°C) and mild and lethal acid conditions (pH 5.0 and pH 4.0) were determined for *A. butzleri* ATCC 49616 and *C. jejuni* NCTC 11168. In addition, it was evaluated whether incubation under the sublethal stress conditions causes specific adaptive responses or cross-protection against subsequent mild or lethal acid stresses in these bacteria. The studies were conducted in broth adjusted to the different conditions and the results were determined by dilution plating. During this study, cross-protection is reported for *A. butzleri* for the first time. Heat stress adapted *A. butzleri* ATCC 49616 (incubated for 2 h at 48°C) were significantly more resistant to subsequent lethal acid stress (pH

4.0) than non-adapted cells at the 1 h time-point ($P < 0.01$). No specific adaptive responses in the bacteria and no cross-protection in *C. jejuni* NCTC 11168 were found.

In conclusion, there is a seasonal peak in the prevalence of *Campylobacter* in Finnish poultry meat products, but otherwise the prevalence is low. *Campylobacter* detection in marinated poultry products indicates that marinating meat might not affect the survival of *Campylobacter*. The PCR assay together with the optimized DNA isolation method is faster than microbiological analyses and could be used for *Campylobacter*-detection in marinated meat samples. Due to the low infective dose of *C. jejuni* in humans and the modest reductions achieved for the strains of *Campylobacter* species studied, the use of UV irradiation or wines and juices as antimicrobial marinade ingredients cannot be recommended as the primary decontamination methods to control *Campylobacter* in broiler meat, but might be used as part of a sequential risk reduction strategy to reduce the counts of *Campylobacter*. The cross-protection phenomenon found for *A. butzleri* ATCC 49616 should be taken into account when designing food preservation strategies containing these conditions. To further evaluate the significance of poultry meat as a source of *Campylobacter* in Finland, their occurrence in the meat products should be quantified. Despite the attempts of this study no sufficiently effective way to reduce the counts of *Campylobacter* in broiler meat was found and further decontamination methods should be studied in the future. Moreover, the cross-protective effect should be investigated further at the gene expression level in order to elucidate the molecular mechanisms behind this phenomenon reported.

1. INTRODUCTION

Campylobacter are recognized as the most frequent cause of foodborne bacterial diarrhoea in humans worldwide (Park 2002, Silva et al. 2011). *Campylobacter* infection (campylobacteriosis) cases are mostly caused by *Campylobacter jejuni*, a bacterium that exists as a commensal organism in the intestinal tract of many birds and mammals (Humphrey, O'Brien & Madsen 2007). The incidence of campylobacteriosis in humans has been steadily increasing since the 1990s and continues to rise in many countries (Baker, Sneyd & Wilson 2007, World Health Organization (WHO) 2011). According to the European Food Safety Authority (EFSA), the incidence ranged from <0.31 to 177.95/100 000 within the populations in different countries in Europe in 2011 (EFSA 2013). In Finland, the number of registered campylobacteriosis cases was 79.29/100 000 in 2011 (4,262 cases; National Institute for Health and Welfare (THL) 2012, EFSA 2013). Thus, *Campylobacter* constitute a major public health problem in humans.

Many studies have shown that the eating and handling of improperly cooked or raw broiler meat is one of the most important sources of human campylobacteriosis (Kapperud et al. 2003, Wingstrand et al. 2006, Lindmark et al. 2009, EFSA Panel on Biological Hazards (BIOHAZ) 2010). Broiler flocks become colonized with *Campylobacter* during rearing via the outside environment or other sources, such as rodents and insects, and the intestines of birds remain highly colonized until slaughter (Berndtson, Danielsson-Tham & Engvall 1996, Rushton et al. 2009, van Gerwe et al. 2009). Contamination of the carcasses and slaughterhouse equipment occurs during slaughtering by leakage of the contaminated faeces from the cloaca and visceral rupture of the ceca still carrying a high *Campylobacter* load (Berndtson et al. 1996, Rosenquist et al. 2006, Perko-Mäkelä et al. 2009). Consequently, broiler meat sold at the retail level is contaminated with different levels of *Campylobacter* (Suzuki, Yamamoto 2009).

In Finland, the consumption of broiler meat has more than doubled over the past decades. Likewise, the popularity of turkey meat products has also been increasing during recent years (http://www.siipi.net). A significant amount (approximately 80%) of Finnish retail poultry meat sold is nowadays marinated with a wide range of flavours, the popularity and variety of which is increasing rapidly every year (Björkroth 2005). Although flavour development, tenderization, and consumer convenience are probably the main reasons for this, marinating could also be used as an antimicrobial treatment on poultry meat (Birk, Knøchel 2009).

Reliable methods for the detection of *Campylobacter* in poultry meat products are of interest to laboratories in routine work and research. Traditional conventional culture methods include enrichment and plating steps followed by isolation of the bacterium and biochemical identification of the isolate. PCR methods have been found to be faster, more specific and sensitive for the detection of *Campylobacter* in meat samples (Denis et al. 2001, Mateo et al. 2005). However, several substances in foods and in marinated broiler meat can be inhibitory for the PCR (Lilja, Hänninen 2001).

Campylobacter is a major public health problem worldwide and many efforts have been directed against finding appropriate intervention methods. Commercially processed broilers go through a variety of steps during processing to reduce microbial contaminants, but *Campylo-*

bacter are still able to survive in the broiler production chain (Suzuki, Yamamoto 2009). Rather than reducing *Campylobacter* prevalence in positive broiler flocks, it is thought the most effective way to control *Campylobacter* in broiler is to reduce their levels on carcasses after evisceration (Hermans et al. 2011). In fact, it has been predicted that a 2 log reduction in the concentration of *C. jejuni* on broiler meat could result in a 30-fold decrease in the number of human campylobacteriosis cases related to the consumption of broiler meat (Rosenquist et al. 2003). However, commercial broiler processing facilities do not currently apply control measures that would completely guarantee the elimination of *Campylobacter* (Oyarzabal 2005).

In addition to *Campylobacter*, the family *Campylobacteraceae* includes a closely related genus *Arcobacter*, which has been linked with bacteraemia and diarrhoea cases in humans (Vandamme et al. 1991, Collado, Figueras 2011). In general, there is no notification of or surveillance for *Arcobacter* as causes of human disease. Thus the estimation of the significance and incidence of human gastroenteritis caused by *Arcobacter* is complicated and probably underestimated (Vandenberg et al. 2004, Lehner, Tasara & Stephan 2005, Kjeldgaard, Jørgensen & Ingmer 2009). In Finland, an *Arcobacter* prevalence of 25.5% in retail broiler meat was found in 2007 (personal communication 2007). However, the ability of *Campylobacter* and *Arcobacter* to persist and survive in the broiler production chain when exposed to multiple stress conditions is not fully understood (Humphrey et al. 2007, Collado, Figueras 2011).

2. REVIEW OF THE LITERATURE

2.1 *CAMPYLOBACTER* SPP.

It is believed that *Campylobacter* species were first described in 1886 by Theodor Escherich who observed spiral shaped non-cultivable bacteria. In 1913, McFadyean and Stockman succeeded in cultivating these bacteria from aborted bovine foetuses (Butzler 2004, Skirrow 2006). Finally, the genus *Campylobacter* was established in 1963 by Sebald and Veron (Moore et al. 2005). Members of the genus are spiral curved, Gram-negative rods. The size of the cells is 0.2 to 0.8 μm wide and 0.5 to 5 μm long. Most *Campylobacter* species are motile by means of a single polar unsheathed flagellum at one or both ends of the cells. *Campylobacter* strains mainly grow under microaerobic (5% O_2, 10% CO_2 and 85% N_2) conditions. All of them grow at 37°C, but 42°C is the optimum growth temperature for the thermophilic species - *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* - mainly responsible for infecting humans. *C. jejuni* is able to grow between 30°C and 47°C, but at 30°C, its growth rate declines rapidly (Vandamme et al. 1991, Butzler 2004, Moore et al. 2005, Jackson et al. 2009). In addition to these fastidious growth requirements, *Campylobacter* are considered to be very fragile and susceptible to many environmental conditions, such as temperature and pH changes, low humidity, the presence of oxygen and UV irradiation. However, they may survive in a viable, but non-cultivable form (VBNC) in the environment (Alter, Scherer 2006). The taxonomy of the genus has been revised many times and to date, the genus has 17 validated species. Most of them are human, animal or zoonotic pathogens (Debruyne, Gevers & Vandamme 2008).

2.2 *CAMPYLOBACTER* IN HUMAN INFECTION

The importance of *Campylobacter* as human pathogens remained undiscovered until the 1970s, mainly because cultivating and isolating these fastidious organisms from faecal samples was so difficult (Butzler et al. 1973, Skirrow 1977). Subsequent intensive research has revealed that *C. jejuni* and *C. coli* are the most common causes of foodborne bacterial gastroenteritis in humans worldwide. The incidence of campylobacteriosis has been steadily rising since 1990s and the incidence continues to increase in many countries (Baker et al. 2007, EFSA 2010b, WHO 2011). The number of confirmed cases of campylobacteriosis in the European Union (EU) has followed a significant increasing trend in the last four years, along with a clear seasonal trend (EFSA 2013). During the last five years in Finland, however, the reported number of infections has been moderately stable (Zoonosis Centre 2012). In 2011, human campylobacteriosis continued to be the most commonly reported zoonosis, with 220,209 confirmed cases in the EU. Within the populations in different European countries, the incidences ranged from <0.31 to 177.95/100 000 in 2011 (EFSA 2013). In Finland, the number of registered campylobacteriosis cases was 79.29/100 000 in 2011 (4,262 cases), with a peak observed in July and August (THL 2012, Zoonosis Centre 2012, EFSA 2013). The wide variation in incidences between countries probably

reflects differences in the healthcare and reporting systems, and in microbiological methods for the detection of *Campylobacter* (Olson et al. 2008, Vally et al. 2009, EFSA 2013). Patients with mild symptoms may also recover without the need for medical care and therefore remain unidentified as campylobacteriosis cases.

Campylobacteriosis is usually a self-limiting disease with an incubation time of one to seven days. The main symptoms are cramps in the abdomen followed by watery, sometimes bloody diarrhoea. General symptoms such as fever, headache and dizziness may also occur (Blaser, Engberg 2008). Common complications in connection with *C. jejuni* infection are musculosceletal symptoms and reactive arthritis occurs in about 4% to 5% of cases (Hannu et al. 2002, Doorduyn et al. 2008, Schönberg-Norio et al. 2010). Infection due to *Campylobacter* may also increase the risk of postinfectious irritable bowel syndrome (DuPont 2008). Guillain-Barré syndrome, a polio-like form of paralysis that can result in respiratory and severe neurological dysfunction and even death, is the most serious, but infrequent later onset complication (Jacobs, van Belkum & Endtz 2008). The infective dose of campylobacteriosis can be as low as 500 cells (Robinson 1981, Black et al. 1988).

2.3 SOURCES OF *CAMPYLOBACTER* IN HUMAN INFECTION

Most campylobacteriosis cases are sporadic or small-scale family outbreaks (Olson et al. 2008). Because the incubation period before the onset of symptoms can be long, it might be difficult to determine the source of infection. However, major sources have been defined. The eating and handling of improperly cooked or raw broiler meat has been shown to be one of the most important sources of human campylobacteriosis (Kapperud et al. 2003, Wingstrand et al. 2006, Lindmark et al. 2009). EFSA also stated that broilers are a major, if not the largest, single source of human infections. The handling, preparation and consumption of broiler meat may account for 20% to 30% of campylobacteriosis cases, while 50% to 80% may be attributed to the broiler reservoir as a whole (EFSA BIOHAZ 2010). Other foods (such as pork, beef and unpasteurized milk), contaminated environmental waters, or direct contact with animals may also be pathways to acquire *Campylobacter* infection (Jacobs-Reitsma, Lyhs & Wagenaar 2008; Fig. 1).

Since 1998, *Campylobacter* have been the most common cause of intestinal infections in humans in Finland. For the majority of the cases, the origin of infection is probably abroad. Even though a high percentage of infections originate from travel abroad and the largest outbreaks have been waterborne, the proportion of infections acquired in Finland in the summer period is considerable (Laine et al. 2011, Zoonosis Centre 2012). It has been estimated that almost one in three *Campylobacter* infections acquired in Finland in summertime are associated with broiler and one in five directly or indirectly with cattle. About half of the *Campylobacter* infections contracted in Finland probably come from other unknown sources. Despite recent suggestions that the importance of broilers in human campylobacteriosis in Finland has been overestimated, broiler meat is still considered to be one of the sources in human campylobacteriosis in Finland (Hakkinen, Nakari & Siitonen 2009, de Haan et al. 2010, Lyhs et al. 2010, Zoonosis Centre 2012). Because the exact sources of human *Campylobacter* infections in Finland are not clear at the moment, further studies are needed in the future.

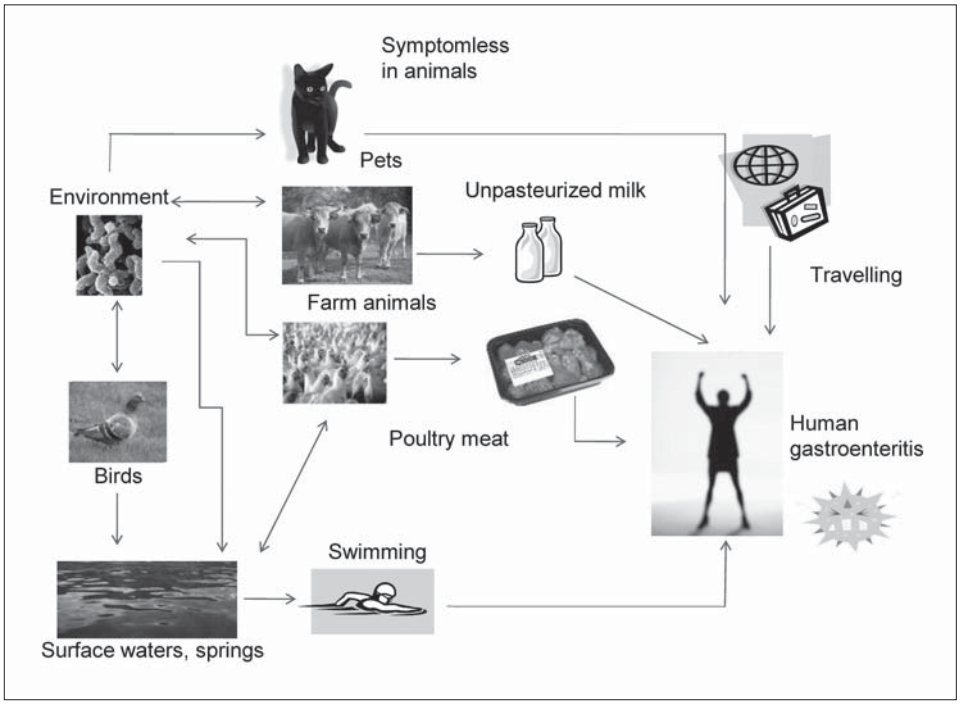

Figure 1. Pathways to *Campylobacter* human infection (according to Perko-Mäkelä 2011).

2.4 *CAMPYLOBACTER* IN BROILER PRODUCTION

This chapter describes the occurrence of *Campylobacter* in different steps of the broiler production chain and indicates the reasons why elevated levels of *Campylobacter* can be recovered from the broiler carcasses and transmitted in the food chain during further processing.

2.4.1 *CAMPYLOBACTER* IN BROILER FARMS

Broiler intestines are a particularly favourable environment for the proliferation of *C. jejuni* and birds carrying *Campylobacter* are asymptomatic colonizers without any clinical signs (Lee, Newell 2006). At the beginning of the rearing period, broiler flocks are free from *Campylobacter*, but after the first colonization (usually at two to three weeks of age), *Campylobacter* spread quickly within the flock. Birds remain highly colonized until slaughter (Berndtson et al. 1996, van Gerwe et al. 2009). The outside environment has been suggested as the ultimate source of colonization for broiler flocks. In addition, many factors - such as adjacent broiler units or other animals, farm workers, drinking water, rodents, wild birds, flies and other insects - may have a role in transmitting *Campylobacter* to broiler flocks (Hald et al. 2004, Bull et al. 2006, Rushton et al. 2009).

The prevalence of *Campylobacter* in broiler flocks varies between different countries. In 2008, approximately 71.2% of broiler batches studied in the EU were estimated to be colonized by *Campylobacter* at the slaughterhouse. The prevalence of *Campylobacter*-colonized broiler

batches among the EU member states varied widely, ranging from as low as 2% up to 100% (EFSA 2010a). In Finland, the prevalence of *Campylobacter* in all slaughtered broiler flocks has been studied since 2004 because of a mandatory *Campylobacter* monitoring programme. Since then, the average prevalence has been 6.5% between June-October. Between July-August higher isolation rates (average 10.5%) and a seasonal peak have been detected. Between November-December and January-May, *Campylobacter* have rarely been detected (Zoonosis Centre 2012). The sharp seasonal variation is observed in other Northern European countries too (Patrick et al. 2004, van Asselt et al. 2008, Jore et al. 2010). The reason for the seasonal variation is unknown, but several factors are probably important. A warmer mean temperature and the moister climate during summertime provide conditions favouring environmental *Campylobacter* survival. Thus, the *Campylobacter* infection pressure from outside the broiler house is higher. The amount of insects, wild birds and rodents around the broiler production environment is also higher during summer (Hald et al. 2004, Rushton et al. 2009, Jore et al. 2010). In the Nordic countries, the cold winters probably decrease the environmental load of *Campylobacter*. In addition, the broiler houses need to be insulated, which also prevents the access of devastators to the houses (Perko-Mäkelä 2011).

2.4.2 *CAMPYLOBACTER* IN BROILER SLAUGHTERHOUSES

The intestinal colonization of broilers with *Campylobacter* during rearing is responsible for the contamination of the carcasses and equipment with *Campylobacter* during slaughtering (Rosenquist et al. 2006, Reich et al. 2008). Carcass contamination occurs especially during scalding, defeathering and evisceration, by leakage of the contaminated faeces from the cloaca and visceral rupture of the ceca carrying a high *Campylobacter* load (Berrang et al. 2001, Boysen, Rosenquist 2009). In addition, carcasses can become contaminated by cross-contamination of *Campylobacter* strains between slaughtered flocks via contacts with surfaces of the slaughter facilities, processing water and air (Peyrat et al. 2008, Perko-Mäkelä et al. 2009). Overall, the whole slaughtering process may reduce the *Campylobacter* contamination level by about 100 to 1,000 times (Rosenquist et al. 2006). Nevertheless, the average prevalence of *Campylobacter* contamination on broiler carcasses worldwide is reported to be in the range of 60-80% (Suzuki, Yamamoto 2009). In 2008, the prevalence in the EU of *Campylobacter*-contaminated broiler carcasses was reported as 75.8% and varied from 4.9% to 100.0%. In Finland it was 5.5% (EFSA 2010a). The counts of *Campylobacter* bacteria on broiler carcasses varied widely also between countries, which might be due to differences in slaughterhouse hygiene and processing practices (Habib et al. 2008, Sampers et al. 2008, EFSA 2010a). In general there was a tendency for high counts in countries with high *Campylobacter* prevalence. Almost half (47.0%) of the carcasses contained less than 10 colony forming units (cfu) of *Campylobacter* per g (cfu/g) and 12.2% contained between 10-99 cfu/g. Higher counts were detected as follows: between 100-999 cfu/g on 19.3%, between 1,000-10,000 cfu/g on 15.8% and more than 10,000 cfu/g on 5.8% of carcasses. The results indicate that elevated levels of *Campylobacter* can be recovered from the broiler carcasses and transmitted in the food chain during further processing (EFSA 2010a).

2.4.3 *CAMPYLOBACTER* IN RETAIL BROILER MEAT PRODUCTS

A large share of retail broiler meat remains contaminated with *Campylobacter*. Table 1 summarizes examples of the prevalence of *Campylobacter* in fresh broiler and turkey meat products sold at the retail level in different countries, as reported in various studies in recent years. It should be noticed that the method used has a large impact on the results shown in Table 1 (the detection limit can vary from 0.1 to 100 cfu/g) and thus the results can not be compared directly. The reported levels of *Campylobacter* in fresh broiler meat products at retail vary between log 1 to log 4 cfu/100 g (or a fillet) of meat, depending on the different studies and methodologies used (Jacobs-Reitsma et al. 2008). *C. jejuni* is usually the dominant *Campylobacter* species isolated from retail broiler meat products worldwide, but the ratio of *C. coli* to *C. jejuni* varies between countries (Suzuki, Yamamoto 2009).

Limited studies have been published on the prevalence of *Campylobacter* in broiler meat at the Finnish retail level. Hänninen et al. (2000) studied the prevalence of *Campylobacter* in broiler meat products in the Helsinki area between June-September from 1996 to 1998 and found from 12% to 21% *Campylobacter* positive samples in each year studied. In summer 2004, the percentages of *Campylobacter* positive fresh broiler and turkey meat at the Finnish retail level were 20.2% and 19.2% respectively (EFSA 2006).

Table 1. Prevalence of thermotolerant *Campylobacter* in fresh chilled broiler or turkey meat products at retail.

Country	Year	Product type	n[a]	% Positive[c]	Enumeration	Reference
USA	2002–2007	Broiler breasts with skin on	6138	49.9	nd[b]	Zhao et al. (2010)
Canada	2001–2004	Broiler legs, thighs, drumsticks, quarters, breasts, halves, wings and backs	1256	59.6	nd	Deckert et al. (2010)
UK	2001–2004	Whole broilers	1675	71.8	nd	Meldrum, Smith & Wilson (2006)
Ireland	2009–2010	Whole broilers and portions	510	84.3	nd	Madden et al. (2011)
France	2000	Broiler legs, drumsticks and breasts with skin, gizzards and escalopes	70	75.7	nd	Denis et al. (2001)
Spain	2002	Broiler necks, drumsticks and wings with skin, carcasses, breasts without skin, livers, gizzards, skin, minced meat	68	79.4	≤ 15 to > 300 cfu	Mateo et al. (2005)
Italy	Not specified	Broiler breasts, wings and legs with skin and bones	104	51.9	nd	Sammarco et al. (2010)
Finland	1996–1998	Broiler legs and breasts	529	17.0	nd	Hänninen et al. (2000)
Sweden	2003	Whole broiler carcasses, breast fillets, drumsticks and spiced/marinated products	397	25.0	≤ 10 cfu/g to 1000 cfu/g	Lindmark et al. (2009)
Denmark	2009–2010	Broiler meat	1469	35.6	nd	Anonymous (2011)
Germany	2003–2004	Skin from broiler legs	140	66.0	Median 2.4 log cfu/g	Scherer et al. (2006)
		Muscle from broiler legs	115	27.0	Median 0.9 MPN/g	
Germany	2004	Surface of skinless and deboned broiler breast fillets	100	87.0	Mean 1903 cfu, median 537 cfu, maximum 38 905 cfu	Luber, Bartelt (2007)
		Deep tissue of skinless and deboned broiler breast fillets	55	20.0	Mean 0.24 cfu, median 0.15 cfu, maximum 0.74 cfu	
Germany	Not specified	Turkey breasts, cutlets, thighs and drumsticks	48	28.0	Mean log 2.1 cfu/g	Atanassova et al. (2007)
		Turkey steaks in various types of marinade	16	6.0		
Belgium	2000–2003	Broiler fillets	975	18.7	nd	Ghafir et al. (2007)
Switzerland	2009–2010	Broiler meat with or without skin	460	55.4	≥ 10 to < 10^4 cfu/g (maximum 8 x 10^3 cfu/g)	Baumgartner, Felleisen (2011)
		Broiler meat preparations (with additives, spices or marinades or processed so that typical texture of meat is maintained)	156	49.4		
Japan	Not specified	Broiler breasts, thighs, wings, livers, gizzards and hearts	170	64.7	nd	Sallam (2007)
Australia (two states)	2005–2006	Whole broilers, wings, thighs, drumsticks and breasts with or without skin	549	87.8	Mean 0.87 log cfu/cm^2	Pointon et al. (2008)
			310	93.2	Mean 0.78 log cfu/cm^2	

[a] Total number of samples examined.

[b] Not determined.

[c] Detection is based on enrichment culture.

2.5 REDUCTION OF *CAMPYLOBACTER* IN THE BROILER PRODUCTION CHAIN

The eating and handling of improperly cooked or raw broiler meat has been shown to be one of the most important sources of human campylobacteriosis (Wingstrand et al. 2006, Lindmark et al. 2009, EFSA BIOHAZ 2010). Although commercially processed broilers go through a variety of steps during processing to reduce microbial contaminants, several studies have revealed that retail broiler meat is frequently contaminated with *Campylobacter* (Suzuki, Yamamoto 2009). The most effective intervention measure to control *Campylobacter* in broiler meat is to reduce *Campylobacter* levels on carcasses after evisceration, rather than reducing the prevalence of positive broiler flocks (Nauta et al. 2009, Hermans et al. 2011). In fact, it has been predicted that a 2 log reduction in the concentration of *C. jejuni* on broiler meat could result in a 30-fold decrease in the number of human campylobacteriosis cases related to the consumption of broiler meat (Rosenquist et al. 2003). Furthermore, a 1-, 2- or 3-log reduction in *Campylobacter* counts on carcasses could reduce the incidence by 48%, 85% and 96%, respectively (Hermans et al. 2011). In general, bacteria are inactivated from foods by heat treatment. Chilling and freezing prevent the growth of bacteria to harmful levels. However, since *Campylobacter* remain a concern even at low levels, their presence in foods at the point of consumption should be prevented (Humphrey et al. 2007). To gain effective results, *Campylobacter* control in broiler should occur throughout the whole production chain from farm to fork. This chapter describes some of the different control methods studied so far.

2.5.1 CONTROLLING *CAMPYLOBACTER* IN BROILER FARMS

Campylobacter control in broiler farms during primary production is expected to significantly reduce the incidence of human campylobacteriosis because the intestine of the living birds is the only growth site for *Campylobacter* in the whole broiler production chain and control would thus indirectly reduce the surface contamination of carcasses (Hermans et al. 2011). Good hygiene and biosecure farming practices aim at preventing the introduction of *Campylobacter* into broiler flocks. During the rearing period, application of specific hygienic measures - such as washing hands and using footbath disinfection before entering the broiler house, changing footwear when entering anterooms and again before entering each separate broiler hall, the use of separate shoes for each broiler house, control of rodents and insects by e.g. fly-screens or by insulated broiler houses, restricted admission, standard hygiene protocol for staff, proper disinfection of the broiler house prior to stocking, and a high standard of cleaning and disinfection of the drinking water equipment - may significantly reduce the risk of *Campylobacter* infections in broiler flocks (Hald, Sommer & Skovgard 2007, Perko-Mäkelä et al. 2009, Hermans et al. 2011). According to EFSA BIOHAZ (2011), however, some control options in primary production - such as the restriction of slaughter age and discontinuing thinning - are directly available from a technical point of view, but interfere strongly with current industrial practices. Perko-Mäkelä (2011) speculated that the reduction of *Campylobacter* contamination at the farm level by a high level of biosecurity control and hygiene may be one of the most efficient ways to reduce the amount of contaminated broiler meat in Finland. Also, other countries that have actively implemented a target strategy to control *Campylobacter* in broilers (for example Denmark, Sweden and Norway) have seen a reduction in the prevalence of *Campylobacter* in broiler flocks and broiler meat (Rosenquist et al. 2009).

There are also different intervention measures aimed at preventing *Campylobacter* transmission in broiler flocks or at reducing the *Campylobacter* load in already colonized birds. Using organic acids, chlorinating, bacteriophages or bacteriocins as feed or water additives and using vaccines or antibodies have been suggested as such measures (Hermans et al. 2011). However, despite all efforts, there is still no effective, reliable and practical intervention measure available to prevent or reduce *Campylobacter* colonization in broiler farms (Hermans et al. 2011). To obtain a further reduction, various decontamination techniques should be used in broiler slaughterhouses.

2.5.2 CONTROLLING *CAMPYLOBACTER* IN BROILER SLAUGHTERHOUSES

In order to reduce the risk represented by *Campylobacter* to consumer health, it is essential to reduce the contamination of broiler carcasses during the slaughtering process. It has been stated that the most effective intervention measure to reduce *Campylobacter* counts on broiler carcasses would be after evisceration (Nauta et al. 2009, Hermans et al. 2011). Scalding can also reduce the *Campylobacter* contamination of carcasses, but cross-contamination can still occur, particularly during the defeathering and evisceration processes and in the chilling water (Rosenquist et al. 2006, Perko-Mäkelä et al. 2009). Overall, the maintenance of slaughter hygiene is of key importance in broiler meat production. Implementation of slaughtering schedules according to the *Campylobacter* contamination status of flocks has also been suggested as an effective risk management solution to control *Campylobacter* at broiler slaughterhouses, especially in low prevalence countries like Finland (Hue et al. 2010, Perko-Mäkelä 2011). *Campylobacter* counts could also be reduced by optimizing the hygienic design of evisceration equipment or by reducing the external surface decontamination of the carcasses throughout the whole slaughtering process.

In an attempt to reduce contamination and improve the shelf life of broiler carcasses, the industry has developed rapid chilling methods. Ideally, after slaughter, broiler carcasses are reduced from body temperature to 4°C or less within 4 to 8 h, depending on carcass weight (Oyarzabal et al. 2010). Immersion chilling, air chilling and evaporative air chilling are common methods used for this in the world. Cryogenic chilling, which uses gaseous refrigerants that evaporate at atmospheric pressure to cause instant cooling or freezing, is a relatively new rapid method for chilling carcasses (El-Shibiny, Connerton & Connerton et al. 2009). Many studies have, however, shown that *C. jejuni* does survive normal refrigerated storage on broiler meat (Solow, Cloak & Fratamico 2003, Pintar et al. 2007, Oyarzabal et al. 2010). El-Shibiny et al. (2009) reported that most of the *C. jejuni* and *C. coli* strains featured an initial fall in their viable counts over 24 h on broiler skin at 4°C. The greatest decline observed in viability over 9 days was 4.3 (± 0.48) log cfu/cm^2 (El-Shibiny et al. 2009). In addition, it has been reported in various studies that the counts of *Campylobacter* in broiler meat decrease rapidly after freezing, but that the bacteria can still be detected after extended storage at -20°C (up to 220 days; Bhaduri, Cottrell 2004, Georgsson et al. 2006, El-Shibiny et al. 2009, Oyarzabal et al. 2010, Sampers et al. 2010). Thus, chilling and freezing maintain a small number of live *Campylobacter*, which means that separately or in combination, refrigeration and freezing are not a substitute for the safe handling and proper storage of broiler (Bhaduri, Cottrell 2004). Nevertheless, channelling *Campylobacter*-positive flocks to frozen products has been suggested as a promising intervention strategy (Rosenquist et al. 2009). According to EFSA BIOHAZ (2011), a 100% risk reduction after slaughter can be reached by irradiation or cooking on an industrial scale if re-contamination is prevented. How-

ever, more than 90% risk reduction can be obtained by freezing broiler carcasses for 2-3 weeks (EFSA BIOHAZ 2011).

Additionally, many other physical decontamination methods for the reduction of *Campylobacter* on fresh broiler carcasses have been studied. For example, treating broiler carcasses with water at 80°C for 20 s followed by crust freezing reduced the numbers of *C. jejuni* by ca. 2.9 log/cm² without extensive degradation of carcass appearance (James et al. 2007). Boysen and Rosenquist (2009) compared the *Campylobacter*-reducing ability of forced air chilling, crust freezing and steam-ultrasound in a plant with naturally contaminated broiler chickens and the mean reductions obtained were 0.44 log cfu per carcass, 0.42 log cfu per samples, and ≥ 2.51 log cfu per carcass, respectively. However, none of these techniques were as effective as freezing based on reductions in *Campylobacter* counts and on adverse effects (Boysen, Rosenquist 2009).

Another possible intervention strategy to control *Campylobacter* in broiler slaughterhouses could be the application of chemical decontamination on carcasses. Acidified sodium chlorite, chlorine, chlorine dioxide, trisodium phosphate, cetylpyridinium chloride, ozone and peroxyacetic acid are the most commonly used antimicrobial substances already used in some US broiler slaughtering plants (Oyarzabal 2005, Boysen, Rosenquist 2009). However, chemical decontamination is subject to approval in the EU and no chemicals are currently approved for use (EFSA BIOHAZ 2011). Thus, they are not further discussed in this summary.

Another possibility to reduce *Campylobacter*-contamination in broiler carcasses at the slaughterhouse could be UV irradiation, which is quite commonly used for the decontamination of packing surfaces or in food processing environments (Corry et al. 1995, Bolder 1997, Dincer, Baysal 2004). The range of UV radiation that is considered to be germicidal against bacteria is between 220 nm and 300 nm (UVC), and generally a wavelength of 254 nm is used for decontamination. This range of UV contains high energy photons that generate pyrimidine dimers and denaturate bacterial DNA, leading to the destruction of bacteria by degradation of the cell walls (Guerrero-Beltrán, Barbosa-Cánovas 2004). The benefits of UV irradiation are that it is a non-thermal, chemical-free process that leaves no residues and the equipment can be easily installed at relatively low cost (Wallner-Pendleton et al. 1994, Wong, Linton & Gerrard 1998, Lyon, Fletcher & Berrang 2007). In the EU, there is currently no legislation prohibiting the use of UV to treat foods, so its use depends on relevant national regulations within individual member states (Haughton et al. 2011). Several studies have been undertaken to investigate the effect of UV irradiation on food items. It reduced *Salmonella* spp. and *Escherichia coli* O157:H7 on fruits and vegetables (Yaun et al. 2004), and other micro-organisms in some liquid foods (Wright et al. 2000, Guerrero-Beltrán, Barbosa-Cánovas 2004). Stermer, Lasater-Smith and Brasington (1987) found that UV lowered the counts of bacteria commonly found on beef, mostly *Pseudomonas*, *Micrococcus* and *Staphylococcus* spp., whereas on broiler skin and carcasses, *Salmonella* Typhimurium was diminished (Wallner-Pendleton et al. 1994, Sumner et al. 1996). In a study by Wong et al. (1998), UV reduced *S.* Senftenberg and *E. coli* on pork muscle and skin. Kim, Silva and Chen (2002) discovered reductions of *Listeria monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium on broiler meat after UV irradiation, as did Lyon et al. (2007) on broiler breast fillets for *L. monocytogenes*. Butler, Lund and Carlson (1987) reported inactivation of *C. jejuni* in liquid samples after UV irradiation.

Activated oxygen is another possible potential agent to decontaminate broiler meat and it could be used in combination with UV irradiation. Activated oxygen can be created from ordinary oxygen through the addition of energy (natural or generated) which changes the momen-

tum of the electrons in the oxygen, or the oxygen becomes ionized. Activated oxygen has oxidizing effects on the ions in lipids and on amino acids in bacteria, which leads to degradation of the cell walls. When the ions react with water vapour, the hydroxyl created destroys the DNA of the bacteria (Seo et al. 2001, Arnold, Mitchell 2002).

2.5.3 CONTROLLING *CAMPYLOBACTER* BY POTENTIAL ANTIMICROBIAL MARINADES

Marinades are complex spiced, acidic water-oil emulsions typically containing salt, sugar, sorbate or benzoate or both. High NaCl concentration, low pH and the addition of different spices to the marinades prevent the growth of spoilage bacteria, thus increasing the shelf-life of meat products (Björkroth 2005). Nevertheless, marinating broiler meat does not decrease pathogenic bacteria such as *Campylobacter* (Perko-Mäkelä et al. 2000). Marinades could, however, potentially be used as antimicrobial treatments on broiler meat by adding substances with antimicrobial properties to them. The antimicrobial property of wine against *C. jejuni* was previously reported by Birk et al. (2007) and Carneiro et al. (2008). Carneiro et al. (2008) suggested that the immersion of food, for example, broiler, in wine as a marinade, leads to a reduction in the number of viable *C. jejuni* cells eventually present, thus lowering the risk of cross-contamination of cooked foods. Several studies have described wines as having antimicrobial properties on also other food pathogens such as *Bacillus* spp., *E. coli* O157:H7, *Listeria* spp., *Salmonella* spp., *Staphylococcus aureus* and *Vibrio parahaemolyticus* (Sugita-Konishi et al. 2001, Just, Daeschel 2003, Møretrø, Daeschel 2004, Liu, Chen & Su 2006, Fernandes et al. 2007, Waite, Daeschel 2007, Hakovirta 2008). McKee et al. (2005) reported that the use of red wine as a rinsing agent of skinless broiler breast meat reduced the total aerobic and coliform counts by 1.5 and 2.2 log cfu/cm^2 respectively. The exact mechanisms responsible for the bactericidal activity of wine are not fully understood, but its low pH, polyphenol compounds, high ethanol content, high organic acid content (such as tartaric, acetic, lactic, malic and citric acids) and sulphur dioxide, either individually or in combination, have been reported responsible for reducing bacterial counts of various food-borne pathogens (Møretrø, Daeschel, 2004, Waite, Daeschel 2007, Carneiro et al. 2008, Ganan, Martínez-Rodríguez & Carrascosa 2009, Birk et al. 2010).

In addition to wines, grape juice has been shown to have bactericidal effects against *Bacillus* spp. and *Listeria* spp., with polymeric phenolic fractions being responsible for antilisterial effects (Rhodes et al. 2006, Hakovirta 2008). Studying antimicrobial properties of wines and juices could help to optimize consumer-friendly *Campylobacter* reduction strategies, which could be used just prior to consumer handling (Birk, Knøchel 2009).

2.5.4 CONTROLLING *CAMPYLOBACTER* IN DOMESTIC KITCHENS

According to epidemiological data, a failure by the consumer to properly prepare or handle contaminated food accounts for a significant proportion of reported foodborne diseases, such as campylobacteriosis (Redmond, Griffith 2003). Commercial broiler processing facilities do not currently apply control measures that completely guarantee the elimination of *Campylobacter* (Oyarzabal 2005). Therefore, the consumer is responsible for using proper food handling techniques. For example, recommendations are given for storing meat at lower temperatures, because it has been reported that approximately 25% of domestic refrigerators may have temperatures exceeding 10°C (Laguerre, Derens & Palagos 2002). In addition, it is important that consumers apply heat treatments that are effective at destroying *Campylobacter*. In general, *Campylobacter* are rather easily inactivated in broiler meat by heat treatments. Gunsen (2008),

for example, reported that baking broiler drumsticks for 3 or 5 min to a core temperature of 80°C and 70°C respectively eliminated all *Campylobacter* cells. Sampers et al. (2010) studied the survival of *Campylobacter* subjected to a heat treatment conforming to consumer-based pan-frying of broiler burgers. After 2 min of pan-frying (internal temperature reached 38°C), *Campylobacter* numbers were found to have declined and after 4 min (internal temperature 57.5°C), they dropped to below detectable levels. However, food may be undercooked or cells in some protected areas of food may survive the normal heating process, leading to ingestion of an infectious dose (Al Sakkaf, Jones 2012). Luber and Bartelt (2007) reported high numbers of *Campylobacter* on broiler meat surface in comparison with the low levels of internal contamination. Therefore, probable cross-contamination from raw contaminated broiler meat during meal preparation seems to be more important than eating improperly heated broiler meat. Cross-contamination of kitchen surfaces or utensils or direct hand to mouth contact after handling raw contaminated broiler meat can occur readily in domestic settings (Jacobs-Reitsma et al. 2008). Therefore, maintaining good hygiene practices when cooking at home, by, for example, using separate knives and cutting boards for preparing broiler meat than for preparing salads, is of prime importance in controlling *Campylobacter* at home.

2.6 DETECTION AND IDENTIFICATION OF *CAMPYLOBACTER*

One of the aims of this study was to compare a PCR assay with the conventional culture method for the detection of *Campylobacter* in poultry meat products. Thus, the two methods are shortly described here.

Faecal samples often contain large numbers of viable *Campylobacter*, thus their detection is easily possible by direct plating on selective media using microaerobic conditions (Fitzgerald, Whichard & Nachamkin 2008). Food products and environmental samples, however, may have only low numbers of stressed *Campylobacter* cells (Jacobs-Reitsma et al. 2008). Several enrichment broths (e.g. Bolton and Preston broth) are available to be used before plating to promote the recovery of damaged cells. During the first stages of enrichment, lower incubation temperatures are often used (4 h at 37°C; Jacobs-Reitsma et al. 2008). A variety of selective agents, such as cefoperazone, amphotericin B, trimethoprim and vancomycin, are included in the broths. Isolation of *Campylobacter* from the enrichment broth is on solid selective agar (e.g. mCCDA; Modified Charcoal Cefoperazone Deoxycholate Agar). Agars contain selective agents and sterile sheep or horse blood or charcoal to neutralise the toxic effects of oxygen and light. Incubation on a solid medium is generally at 41.5°C for 48 h. All incubations are performed under microaerobic conditions for which many atmosphere systems are available (Fitzgerald et al. 2008, Jacobs-Reitsma et al. 2008). In case of a presumptive positive result, further identification is performed microscopically (Gram-negative, motile, typical morphology) and the absence of aerobic growth. A number of biochemical tests, including catalase, oxidase and hippurate tests may be done, but the results are often confusing (Debruyne et al. 2008). To study the presence of less common *Campylobacter* species (non-*jejuni*, non-*coli*), appropriate cultivation conditions need to be applied, such as membrane filtration, special atmospheric and temperature conditions, prolonged incubation, or subsequent plating on non-selective media (Debruyne et al. 2008).

In recent years, a wide range of nucleic acid-based methods, particularly PCR methods, has become available to detect and identify *Campylobacter*. The 16S and 23S rRNA ribosomal genes are two widely used targets for the design of species-specific tests (Fitzgerald et al. 2008).

Most of the published PCR-based detection methods require an initial enrichment step (Jacobs-Reitsma et al. 2008). The advantages of PCR are that it can quickly detect and identify *Campylobacter* to the species level (even simultaneously by a single assay) and the method is relatively uncomplicated to use. In meat samples also, PCR methods have been found to be faster, more specific and sensitive for the detection of *Campylobacter* (Denis et al. 2001, Mateo et al. 2005). The disadvantages of PCR are the limited availability of the technology and the fact that it is expensive, labour intensive and does not provide an isolate for further identification or typing (Kulkarni et al. 2002). Sample preparation is crucial in PCR, because the presence of inhibitory compounds coming from the sample may affect the PCR reaction and give false-negative results. The use of an internal standard controls the PCR reaction and increases its reliability (Denis et al. 2001). Another important aspect is that the PCR method may detect dead as well as viable bacteria (Waage et al. 1999).

2.7 *ARCOBACTER* SPP. AND HUMAN AND ANIMAL INFECTIONS

In the late 1970s, aerotolerant *Campylobacter*-like microorganisms were isolated from aborted bovine and porcine foetuses (Ellis et al. 1977, Neill, Ellis & O'Brien 1979). These organisms were later classified in the genus *Arcobacter* belonging to the family *Campylobacteraceae* (Vandamme et al. 1991). The morphological characteristics of *Arcobacter* are similar to those of *Campylobacter*; i.e. Gram-negative, spirally curved rods, generally 0.2-0.9 μm wide and 0.5-3 μm long, non-spore forming and motile by means of an unsheathed single polar flagellum at one or both ends of the cells. Distinctive features that differentiate *Arcobacter* from their close phylogenetic relative *Campylobacter* are their ability to grow aerobically over a wider temperature range (10-42°C; optimally at 30°C). Like *Campylobacter*, *Arcobacter* grow optimally under microaerobic conditions (Ho, Lipman & Gaastra 2006, Snelling et al. 2006, Kjeldgaard et al. 2009). *Arcobacter butzleri* is the most important and prevalent species of the genus being classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF 2002) and as a significant zoonotic pathogen (Cardoen et al. 2009).

So far, only the species *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been isolated from human and animal infections (Collado, Figueras 2011). In humans, *A. butzleri*, and more rarely *A. cryaerophilus* and *A. skirrowii*, have been associated with diarrhoea (Vandenberg et al. 2004, Prouzet-Mauleon et al. 2006, Samie et al. 2007) and *A. butzleri* and *A. cryaerophilus* with bacteraemia (Yan et al. 2000, Woo et al. 2001, Lau et al. 2002). Very recently, *A. butzleri* was found to be the etiological agent of traveller´s diarrhoea (Jiang et al. 2010). In general, there is no notification of or surveillance for *Arcobacter* as causes of human disease. Thus the estimation of the significance and incidence of human gastroenteritis caused by *Arcobacter* is complicated and probably underestimated (Vandenberg et al. 2004, Lehner et al. 2005, Kjeldgaard et al. 2009). Various media and procedures have been used to isolate *Arcobacter* from different samples, but a standardized reference method has so far not been proposed (Collado, Figueras 2011). *Arcobacter* infection of humans probably occurs by the oral route via contaminated food or water (Ho et al. 2006, Collado, Figueras 2011). Like for *Campylobacter*, a high prevalence of *Arcobacter* is observed on broiler meat at the retail level (highest prevalence) and in many other food products of animal origin (pork, beef, lamb, shellfish; Rivas, Fegan & Vanderlinde 2004, Collado et al. 2009). It has been indicated that the contamination of meat products by *Arcobacter* prob-

ably occurs when the faeces of contaminated animals come into contact with carcasses during the slaughtering process (Aydin et al. 2007). In fact, it has been suggested that poultry could be a natural reservoir of *Arcobacter* (Atabay et al. 2008, Ho, Lipman & Gaastra 2008, Lipman, Ho & Gaastra 2008). Apart from in broiler and other meat, *Arcobacter* have been detected in various types of water, including surface water, ground water, raw sewage, drinking water reservoirs and water treatment plants (Lehner et al. 2005, Collado, Figueras 2011). The use of these contaminated water sources could result in *Arcobacter* animal colonization and human illness (Snelling et al. 2006).

In different farm animals, such as cattle, pigs and poultry, *Arcobacter* have frequently been isolated from the intestinal tracts and faecal samples, but it apparently has the capacity to cause disease in some animals (Ho et al. 2006). The most serious effects of *Arcobacter* in these farm animals include abortions, mastitis and diarrhoea (On et al. 2002, Van Driessche et al. 2003).

2.8 SURVIVAL OF STRAINS OF *CAMPYLOBACTER* AND *ARCOBACTER* SPECIES UNDER SPECIFIC STRESS CONDITIONS

Not enough is known about how *Campylobacter* and *Arcobacter* are able to persist and survive when exposed to multiple stress conditions in the broiler meat production chain (Humphrey et al. 2007, Collado, Figueras 2011). This study concentrated on studying the effects of heat, cold and acid stresses on the survival of these bacteria in broth, thus other stresses are not discussed in this summary. Another aim was to evaluate whether adaptations to sublethal heat, cold or acid stresses improve the survival of *C. jejuni* and *A. butzleri* under subsequent acid stress. That is why the last chapter of this review focuses on the stress adaptation phenomenon. Previously, some research on the effectiveness of different temperature and acid treatments on the survival of *Campylobacter* and *Arcobacter* has been conducted. It is important to note that the survival of bacteria under different stresses depends on the sources, growth conditions and growth phases of the bacteria studied. The medium in which the experiments were carried out also influences the results (Murphy, Carroll & Jordan 2003, Murphy, Carroll & Jordan 2005).

2.8.1 HEAT STRESS

The strains of *Campylobacter* and *Arcobacter* species are rather easily inactivated by heat treatments. Decimal reduction times (*D*-values) for *C. jejuni* were determined as 15.2, 4.90, 1.71, 0.64 and 0.25 min in broth at 49°C, 51°C, 53°C, 55°C and 57°C, respectively (Blankenship, Craven 1982). In another study, the *D*-values for *C. jejuni* were reported to range from 0.88 to 1.63 min at 50°C in broth (ICMSF 1996). Correspondingly, *C. jejuni D*-values ranged from 228 to 1.5 s at temperatures from 51.5°C to 60°C in broth (Al Sakkaf, Jones 2012). Recently, initial concentrations of *C. jejuni* ranging from 7.63 to 8.16 log cfu/ml were reduced to counts of between 3.0 and 5.54 log cfu/ml after a 5-min challenge at 55°C in broth (Habib, Uyttendaele & De Zutter 2010). Yang, Li and Johnson (2001) found that reductions in *C. jejuni* counts were 1.5 and 6.2 log cfu/ml after a 5-min treatment in 50°C and 60°C scalding water, respectively. At 55°C, almost all the cells died and the reduction was 5 logs more than at 50°C. At 60°C, the whole population was killed in 1 min (Yang et al. 2001). Hilton et al. (2001) found *D*-values for stationary phase *A. butzleri* cells in broth to be 0.4 min at 55°C and 1.7 min at 50°C. In another study, the *D*-values for *A. butzleri* in broth ranged from 5.81 min at 50°C to 0.4 minutes at 55°C (D'Sa, Harrison 2005).

Phillips and Duggan (2002) investigated the sensitivity of *A. butzleri* to 10-min treatments at 60°C and 50°C in broth. After treatment at 60°C, they detected no cfus' over the subsequent 24 h incubation at 30°C, but after 50°C, however, cells were detected after 24 h incubation at 30°C (12.2% survival rate; Phillips, Duggan 2002). Van Driessche and Houf (2008), incubated *Arcobacter* in water at 52°C, 56°C and 60°C (to represent scalding temperatures), where the bacteria surprisingly survived several minutes of incubation (30, 18 and 4 min, respectively). Ho et al. (2008) also indicated that some *Arcobacter* were able to survive in tap water at 52°C for 3 min. Based on the experiments in water, *Arcobacter* seem more heat resistant than *C. jejuni*, but the conclusion should be drawn with caution, because it is based on only two studies involving a limited number of *Arcobacter* strains (Cervenka 2007).

2.8.2 COLD STRESS

The effects of cold storage on *Campylobacter* and *Arcobacter* strains have been reported as quite similar. Hilton et al. (2001) found that *A. butzleri* was able to survive for at least 3 weeks at 4°C in broth, but the counts gradually decreased (log 4) during storage. Correspondingly, Habib et al. (2010) reported that after 7 days at 4°C in broth, viable counts of *C. jejuni* declined by less than 10% to ~20% of its initial concentrations. In the study by Hilton et al. (2001), freezing in -20°C broth caused a 2 log decrease in *A. butzleri* viability after only 24 h storage, after which viability remained constant. The decrease during the first 24 h of freezing appears to be consistent with results for *C. jejuni* in liquid media (Chan et al. 2001, Habib et al. 2010). In the study by Hilton et al. (2001), *A. butzleri* was able to survive at -20°C in broth for at least 3 weeks. D'Sa and Harrison (2005) on the other hand, observed that *A. butzleri* was able to survive prolonged incubation at -20°C (6 months) in a medium with a 0 to 1.5 log decrease in cell numbers. In water at 4°C and 7°C, *A. butzleri* can remain viable for an extended period of time (98 days; Van Driessche, Houf (2008). In addition, Kjeldgaard et al. (2009) observed less than a one log reduction in *A. butzleri* counts in chicken meat juice (CMJ) medium after 77 days incubation at 5°C, which was a longer period of time than that observed for *C. jejuni* in the same study. In the study by Cools et al. (2003), *C. jejuni* survived in pure drinking water at 4°C for 33 days. In addition, Yang et al. (2001) found that the counts of *C. jejuni* did not change significantly in chilled water or on broiler skins during chilling after 50 min treatment at 2°C. Overall, it has been reported that *C. jejuni* survive better at 4°C in various biological milieux than at 25°C (Murphy, Carroll & Jordan 2006). Recently, Lu et al. (2011), however, showed a 2 log decrease in *C. jejuni* numbers after 12 days in 4°C bottled drinking water and it survived poorly after 20 days incubation at -18°C (~ 6 log reduction).

2.8.3 ACID STRESS

Acid stress in this context means stress caused by different pH levels. Studies have shown that the responses of *Arcobacter* and *Campylobacter* strains to different pH levels are quite comparable. *Campylobacter* strains are generally recognized as being sensitive to low pH values. They grow optimally at pH 6.5-7.5 with a minimum pH value at 4.9 and a maximum at 9.0, but do not grow at pH 4.0 (Park 2002, Chaveerach et al. 2003, Jackson et al. 2009, Silva et al. 2011). Similarly, *A. butzleri* have been shown to be able to grow between pH 5.0 and 8.5 (optimally between pH 6.0-8.0), with little if any growth below pH 5.0 and not surviving at pH 4.0 (Hilton et al. 2001, D'Sa, Harrison 2005, Cervenka 2007). However, the survival of bacteria does not

depend only on the pH value, but also on the acid used, as some acids are more effective against these bacteria even though their pH is the same. For example, pH adjusted by the addition of tartaric acid was more inhibitory for *Arcobacter* than the same pH adjusted by lactic acid (Cervenka 2007). The composition of the test medium also influences inactivation during acid stress. Shaheen, Miller and Oyarzabal (2007), for example, found Brucella broth to be more protective than Tryptic soy broth for *C. jejuni* cells at pH 4.0.

2.8.4 STRESS ADAPTATION

Many bacteria have the ability to adapt to stressful conditions. This ability may later protect them against the same type of stress or different types of stresses, phenomena known as specific adaptive response or multiple adaptive response, also termed cross-protection (Xu, Lee & Ahn 2008). Previously, specific adaptive responses and cross-protection against different stress conditions have been reported for *C. jejuni*. Murphy et al. (2003) described the abilities of *C. jejuni* to survive better under lethal pH conditions after initial adaptation to mild acid stress or aerobic stress, or both. Ma, Hanning and Slavik (2009) demonstrated that *C. jejuni* cells adapted to acid, acid and aerobic, or starvation conditions were able to better withstand further acid challenges than non-stressed cells. An adaptation to heat after prior heat stress has also been described in this organism (Mihaljevic et al. 2007). In addition, it has been shown that starved *C. jejuni* cells were able to withstand heat stress (Klančnik et al. 2009). Cross-protection at the gene expression levels was reported by Reid et al. (2008), where genes involved in heat stress response in *C. jejuni* were upregulated in response to acid stress, too. These kinds of stress adaptation phenomena might affect the ability of *Arcobacter* and *Campylobacter* to survive in the food chain. To the best of the author's knowledge, however, no comparative *Arcobacter* studies have been published to date.

3. AIMS OF THE STUDY

The specific aims of this study dealing with survival and reduction of strains of *Campylobacter* species in broiler meat were:

I. To determine the prevalence of *Campylobacter* in marinated and non-marinated poultry meat products at the Finnish retail level.

II. To compare a PCR assay with the conventional culture method for the detection of *Campylobacter* in poultry meat products and to modify a commercial DNA isolation method for marinated products.

III. To study the effects of UV irradiation to reduce *C. jejuni* in broiler meat.

IV. To study the potential of wines and juices to be used as antimicrobial marinade ingredients to control *Campylobacter* in broiler meat.

V. To evaluate the survival of *A. butzleri* and *C. jejuni* in heat, cold and acid stress conditions and to determine specific adaptive responses and cross-protective effects of temperature and acid stresses on *A. butzleri* and *C. jejuni*.

4. MATERIALS AND METHODS

4.1 POULTRY MEAT SAMPLES (I-III)

The poultry meat samples used in studies I-III were bought from a local retail shop, except in study II, where the broiler carcasses and broiler fillets with or without skin were obtained from a local broiler slaughterhouse. The poultry (broiler and turkey) meat samples described for study I in Table 2 were used for the detection of *Campylobacter* in poultry meat products at the Finnish retail level. The samples described for study II were used for inoculation with a *C. jejuni* strain and for studying the effects of UV irradiation to reduce the counts of the *C. jejuni* strain in broiler meat or for the sensory analyses of broiler meat after UV treatment. Broiler meat samples described for study III were used for inoculation with different strains of *Campylobacter* species and for investigating the effects of wines on the survival of these *Campylobacter* strains in broiler meat (Table 2).

Table 2. Description of poultry (broiler and turkey) meat samples used in studies I-III.

Sample description	n[1]	Study
Total broiler meat products	136	I
Marinated	95	
Non-marinated[2]	41	
Total turkey meat products	56	
Marinated	39	
Non-marinated[2]	17	
Total marinated mixed broiler-turkey meat products	2	
Total poultry meat products[3, 4]	194	
		II
Skin pieces (2 x 2 cm) cut from broiler leg meat	150	
Meat pieces (2 x 2 cm) cut from boneless, skinless broiler breast fillets	150	
Fresh broiler carcasses	48	
Broiler fillets with skin	60	
Broiler fillets without skin	60	
Portions of 10 g cut from broiler breast fillets	48	III

[1] Number of samples used in the corresponding study.

[2] Natural, lightly salted or spiced products.

[3] Slices, barbecue sticks, breast fillets, fillet steaks, breasts, legs, drumsticks or wings including bones and skin.

[4] All were packed in Finland, but in 11, nine and two samples, the meat originated from Denmark, Brazil and France respectively.

4.2 BACTERIAL STRAINS (I-IV)

Table 3 shows the bacterial strains used to validate the specificity of the C412F-16S rRNA-campR2 primer set used in study I. The other bacterial strains used in studies I-IV are described in Table 4. The strains were maintained at -80°C in Brain Heart Infusion (BHI) broth (Difco, Detroit, MI, USA) containing 20% glycerol (study I), or at -75°C in Brucella broth (Scharlau Chemie, Barcelona, Spain) containing 15% glycerol (studies II and III) or in freezing broth containing distilled water supplemented with 0.5% (w/v) sodium chloride (Merck, Darmstadt, Germany), 0.5% (w/v) meat extract (Merck), 1% (w/v) peptone from casein (Merck), 2% (w/v) D(+)-Glucose (Merck), 25% glycerol and 20% horse serum (study IV).

Table 3. List of strains used for validation of specificity of the C412F-16S rRNA-campR2 primer set in study I.

Species	Strain	Species	Strain	Species	Strain
C. jejuni	CCUG 11284	*C. coli*	DCC 51	*C. helveticus*	CCUG 34016
C. jejuni	CCUG 24567	*C. coli*	DCC 28	*C. hyointestinalis*	CCUG 14169
C. jejuni	CCUG 10940	*C. coli*	DCC 18	*C. hyointestinalis*	CCUG 34538
C. jejuni	CCUG 12778	*C. upsaliensis*	CCUG 23626	*C. sputorum*	CCUG 37579
C. jejuni	DCC*42	*C. upsaliensis*	CCUG 14913	*C. concisus*	CCUG 13144
C. jejuni	DCC 43	*C. upsaliensis*	CCUG 24571	*C. curvus*	CCUG 13146
C. jejuni	DCC 44	*C. upsaliensis*	CCUG 24803	*C. mucosalis*	CCUG 6822
C. jejuni	DCC 45	*C. upsaliensis*	CCUG 23017	*C. fetus*	CCUG 6825A
C. jejuni	DCC 47	*C. upsaliensis*	CCUG 20818	*A. cryaerophilis*	CCUG 17801
C. jejuni	DCC 48	*C. lari*	CCUG 23947	*A. skirrowii*	CCUG 10374
C. jejuni	DCC 49	*C. lari*	CCUG 20575	*A. butzleri*	CCUG 30485
C. jejuni	DCC 52	*C. lari*	CCUG 18267	*Helicobacter pylori*	DCC 35
C. jejuni	DCC 22	*C. lari*	CCUG 15035	*Helicobacter pullorum*	DCC 53
C. jejuni	DCC 27	*C. lari*	CCUG 12774	*Enterococcus faecalis*	CCUG 19916
C. jejuni	DCC 34	*C. lari*	CCUG 18294	*Escherichia coli*	CCUG 17620
C. jejuni	DCC 40	*C. lari*	DCC 50	*Streptococcus aureus*	CCUG 17621
C. jejuni	DCC 41	*C. lari*	DCC 29	*Staphyloccus bovis*	CCUG 17828
C. coli	CCUG 11283	*C. lari*	DCC 33	*Salmonella* Typhimurium	DVI-Å¨19
C. coli	CCUG 33450	*C. helveticus*	CCUG 30682	*Salmonella* Enteritidis	DVI-Å20
C. coli	DCC 36	*C. helveticus*	CCUG 30683	*Proteus mirabilis*	CCUG 34293
C. coli	DCC 37	*C. helveticus*	CCUG 30563	*Bordetella bronchiseptica*	DVI-Å50
C. coli	DCC 38	*C. helveticus*	CCUG 30564	*Citrobacter freundii*	DVI-Å22
C. coli	DCC 39	*C. helveticus*	CCUG 30565		
C. coli	DCC 46	*C. helveticus*	CCUG 30566		

*DVI culture collection.

¨DVI-Å in house reference strain.

Table 4. List of bacterial strains used in studies I-IV.

Species	Strain	Origin	Study
C. jejuni	EELA 49[1]	Finnish broiler carcass at slaughterhouse	I
C. jejuni	E1 1347[1]	Broiler caecum content at slaughterhouse	II
C. jejuni	RefCJ (NCTC 11168)[2]	Clinical human isolate	III,IV
C. jejuni	RetCJ29[1]	Finnish honey-marinated retail turkey meat	III
C. coli	RetCC27[1]	Brazilian honey-marinated retail broiler meat	III
C. jejuni	SlaCJ26[1]	Turkey caecum content at slaughterhouse	III
A. butzleri	ATCC 49616[3]	Clinical human isolate	IV

[1] Identified according to a modified method of NCFA (2007).

[2] Obtained from National Collection of Type Cultures, Health Protection Agency, Centre for Infections (London, UK).

[3] Obtained from LGC Standards (Teddington, UK).

4.3 DETECTION OF *CAMPYLOBACTER* IN POULTRY MEAT SAMPLES (I)

4.3.1 SAMPLE COLLECTION (I)

In study I, 194 raw chilled poultry (broiler and turkey) meat products were randomly collected between January and September 2006 from different local retail shops in a defined area in Western Finland (Table 2). All samples were kept at 4°C until being analysed within 24 h of purchase. Between January and June, 10 samples were analysed once a month and from July to September, 15 samples were analysed three times a month.

4.3.2 CULTURE METHOD FOR DETECTION OF *CAMPYLOBACTER* IN POULTRY MEAT SAMPLES (I)

In study I, microbiological analyses of the samples were based on a modified method of the Nordic Committee of Food Analyses (NCFA 2007). Each sample was aseptically removed from the package and placed in a Stomacher bag (Seward, Worthing, UK). Equal amounts of a weighed sample and Buffered Peptone Water (BPW; LabM, Lancashire, UK) were mixed with a minimum amount of 300 g of meat in 300 ml of BPW. The bag was shaken manually for 3 min. For enrichment, 25 ml of the suspension was re-suspended in 225 ml of Bolton broth (LabM) with 5% lysed horse blood and selective supplement (LabM), and incubated at 42°C for 24 h under microaerobic conditions generated by CampyGen (Oxoid, Basingstoke, UK). Sterile Bolton broth was used as a negative control. A loopful of the enrichment culture (10 µl) was streaked onto mCCDA prepared from Campylobacter Blood-Free Selective Agar Base (Oxoid) and CCDA Selective Supplement (Oxoid), and incubated at 42°C for 48 h under microaerobic conditions. Presumptive *Campylobacter* colonies on mCCDA were further identified according to the NCFA (2007) method. To test their ability to grow aerobically, the colonies were subcultured onto Casein-Peptone Soymeal-Peptone agar (CASO; Merck) with 5% bovine blood and incubated aerobically at 37°C for 24 h. Strains were stored at -80°C in Brucella broth containing 15% glycerol.

4.3.3 PCR METHOD FOR DETECTION OF *CAMPYLOBACTER* IN POULTRY MEAT SAMPLES (I)

For the PCR sample in study I, 1.5 ml of the rinsing fluid was centrifuged at 1,000 rpm for 8 min at 4°C. The middle aqueous layer was removed carefully to avoid any fat and placed into an unused Eppendorf tube. After centrifugation at 13,000 rpm for 8 min at 4°C, the supernatant was removed. For the PCR of the enriched sample, 1 ml of enrichment culture was collected after 24 h incubation. It was centrifuged at 13,000 rpm for 8 min at room temperature and the supernatant was removed. The pellets were frozen at -70°C.

DNA ISOLATION

DNA isolation from the frozen pellet was carried out using a DNA isolation kit, MagneSil KF Genomic System (Promega, Madison, WI, USA), with a Dynal MPC-S magnetic stand (Dynal Biotech, Oslo, Norway). The supplier's instructions were modified and optimized for DNA isolation by hand using a magnetic stand. A 200 µl lysis buffer and 75 µl magnetic beads were added to an Eppendorf tube containing the pellet. The mixture was vortexed vigorously four times during a 5-min period at room temperature before placing the tube in a magnetic stand with the magnet for 30 s. The magnet was taken out after the liquid was removed from the tube. The particles were washed twice with 185 µl of salt washing buffer and twice with 200 µl of ethanol washing buffer. The tube was then placed in a 72°C heat block for 5 min with an open lid for ethanol dehydration. The particles were re-suspended in 100 µl of sterile water and replaced in a 72°C heat block for another 5 min with the lid closed. The tube was vortexed and placed in the magnetic stand for 30 s. The liquid was removed from the tube and frozen at -20°C.

PCR ASSAY AND AMPLIFICATION

The detection of *Campylobacter* in the samples was based on amplification of the 16S rRNA gene using two sets of oligonucleotide primers. The first set was a new combination of primers: C412F 5'-GGA TGA CAC TTT TCG GAG C-3' (from Linton, Owen & Stanley 1996) and 16S rRNA-campR2 5'-GGC TTC ATG CTC TCG AGT T-3' (from Lund et al. 2004). The strains used to validate the specificity of the C412F-16S rRNA-campR2 primer set are listed in Table 3. For testing the specificity of the primers used in the assay, DNA was isolated directly from the storage medium by centrifugation of 0.1 ml of the medium at 15,870 rpm for 7 min and then the pellet was subjected to DNA isolation as described before. The second set was MD16S1, 5'-ATC TAA TGG CTT AAC CAT TAA AC-3' and MD16S2, 5'-GGA CGG TAA CTA GTT TAG TAT T-3' as described by Denis et al. (1999). For detection of the internal control the set of primer YersF8 5'-CGA GGA GGA AGG GTT AAG TG-3' and YersR10 5'-AAG GCA CCA AGG CAT CTC TG-3' was used (Gibello et al. 1999). All primers were synthesized by Oligomer Oy (Helsinki, Finland).

The PCR conditions used are described by Lund et al. (2003) with a few modifications. Briefly, the PCR amplification was performed in 50 µl volumes containing 5 µl of the DNA, 25 µl of a PCR master mix (Promega, Madison, WI, USA), 1 µl of a 25mM $MgCl_2$ solution (Sigma-Aldrich, Saint Louis, MO, USA), 0.5 µl of a 10 mg/ml Bovine Serum Albumin (BSA) solution (Sigma-Aldrich), 20 pmol of each of the *Campylobacter* primers and 5 pmol of the internal control primers. The PCR was performed in a Peltier Thermal Cycler (PTC-200; MJ Research Inc., Watertown, MA, USA) and the conditions were one cycle of 95°C for 2 min, 58°C for 1 min,

72°C for 1 min, followed by 34 cycles of 95°C for 15 s, 58°C for 40 s and 72°C for 40 s. The last elongation step lasted 5 min. Strain EELA 49 (Table 4) was used as a positive control in the PCR assays and sterile water as a negative control. An internal control (DNA isolated from *Yersinia ruckeri*; obtained from Marianne Lund, National Veterinary Institute, Technical University of Denmark, Århus, Denmark) was also added to the PCR mastermix as in Lund et al. (2004) and Lund and Madsen (2006).

The PCR product was loaded onto a 2% agarose gel (1.35% SeaKem LE Agarose and 0.65% NuSieve GTG Agarose, Cambrex Bio Science, Rockland, ME, USA) containing 0.1 g/ml ethidium bromide. A DNA molecular weight marker 100 base pair (bp) low ladder (Sigma-Aldrich) was included in each gel. The gel was photographed under UV light (Alpha DigiDoc, Alpha Innotech, San Leandro, CA, USA). The PCR reaction for each sample was performed 1-3 times with each primer set and considered positive if both primer sets gave a distinct band of the right size (857 bp) or at least one primer set gave a positive reaction twice. Samples with no internal control band were run again using a tenfold dilution of DNA.

4.3.4 COMPARISON OF THE DETECTION LIMIT BETWEEN THE CULTURE AND PCR METHOD (I)

To compare the detection limit between the culture and PCR method in study I, a tenfold dilution series of a *C. jejuni* broth culture was used to determine the detection limit of the culture and the PCR method. For counting the cfu of the stock solution, 100µl of each dilution from 10^{-1} to 10^{-7} was plated out. Seven samples of 100 g broiler meat slices and 42 g of plain marinade were placed in a Stomacher bag. One ml of each dilution of *C. jejuni* broth culture was mixed with 100 ml of BPW and this mixture was added to the samples. All samples were subjected to both direct and enrichment culture and PCR methods as described above. This procedure was repeated once.

4.4 DETERMINATION OF THE EFFECTS OF UV IRRADIATION TO REDUCE *C. JEJUNI* ON AGAR PLATES AND ON BROILER MEAT, SKIN AND CARCASSES (II)

A *C. jejuni* strain E1 1347 was used in study II (Table 4). The cells from the frozen stock culture of the strain were plated onto mCCDA agar and incubated at 42°C for 24-48 h under microaerobic conditions obtained by GasPak EZ Campy Container System Sachets (260680, BD, NJ, USA). To determine the effects of UV irradiation to reduce the *C. jejuni* strain, the studies were conducted on the surfaces of agar plates and on broiler meat, skin and carcasses.

4.4.1 AGAR PLATES (II)

For preparation of agar plates in study II (Trypticase soy agar II with 5% horse blood; TSA, 212099, BD), the resulting growth of the *C. jejuni* strain used was first suspended into 10 ml physiological saline. One ml of this suspension was serially diluted in 9 ml of physiological saline (from 10^{-1} to 10^{-6}) and 12 separate TSA agar plates from each dilution were prepared by spreading 100 µl aliquots on the plates. Three of the 12 spread plates prepared from each dilu-

tion were not UV-treated (controls) and the other nine were subjected without a lid to the three different doses of UV light studied (three plates/dose). The UV doses studied were 9.4, 18.8 and 32.9 mWs/cm^2 based on the UV equipment manufacturers' suggestion. The UV treatments of agar plates were conducted using an UV irradiator (BIOCID 72 IP67, Oy BIOCID Ltd, Vantaa, Finland) with four lamps generating 254 nm wavelength and 5.5 W UV effect per lamp. After treatments, the agar plates were incubated at 42°C for 48 h under microaerobic conditions. The reductions in single experiments on agar plates were calculated based on the average cfu/ml from the triplicate spread plates, without and after the UV treatments. The final log reductions were based on the averages from the results of the test replications and calculated by using the formula: log reduction = $\log_{10}$ initial concentration – $\log_{10}$ final concentration.

4.4.2 BROILER MEAT AND SKIN (II)

For broiler meat and skin sample preparation in study II (10 samples per experiment), the samples were aseptically cut into 2 x 2 cm pieces and placed flattened out on the bottom of small, sterile Petri dishes. Before inoculation the sample surfaces were flamed with a Bunsen burner for about 5 s and cooled for 5 min. For inoculation of the samples, the resulting growth of the *C. jejuni* strain used was first suspended into 10 ml physiological saline. Then the samples were inoculated by spreading a 100 µl aliquot of the suspension evenly over the entire surface of each sample and allowed to dry for 15 min. In each experiment with broiler meat or skin, five broiler meat or skin samples were not UV-treated (controls) and five were UV-treated using the same UV irradiator and doses as in the studies on agar plates. One dose was studied per experiment. After UV treatments to determine bacterial counts, each sample surface was swabbed with five sterile pre-moistened cotton swabs which were placed into 5 ml of physiological saline, allowed to soak for 5 min and then removed. One ml of the suspension from each sample was serially diluted in 9 ml of physiological saline and three spread plates (TSA agar) were prepared from each dilution. The final log reductions were calculated as described above. The tests on broiler meat or skin were replicated five times for each three UV doses. All broiler meat and skin samples (20 g of meat per package) of study II were determined to be free of *Campylobacter* by the enrichment PCR method as in study I.

4.4.3 BROILER CARCASSES (II)

For inoculation of broiler carcasses in study II, the resulting growth of the *C. jejuni* strain was first suspended into 10 ml BPW of which 1 ml was re-suspended into 99 ml sterile BPW. The 100 ml BPW bacterial suspensions were used for inoculations of broiler carcasses individually. The carcasses were shaken manually with the suspension for 3 min in a big Stomacher bag (BA6042, LabM), aseptically removed and allowed to dry for 15 min. In one experiment, eight broiler carcasses were used. Two of the carcasses were not UV-treated (controls) and the other six were subjected to the three doses of UV light studied (two carcasses/dose). The UV doses studied on broiler carcasses were 10.8, 18.0 and 32.4 mWs/cm^2 based on the UV equipment manufacturers' suggestion. For the studies, a UV chamber (Fig. 2) was constructed by Oy BIOCID Ltd. Three UV irradiator units (BIOCID 110 IP55, Oy BIOCID Ltd) were mounted inside the chamber walls (made of stainless steel) so that the carcasses received UV light from three sides. Each of the units contained four lamps generating 254 nm wavelength and 16.5 W UV effect per lamp. The effects of UV irradiation on the survival of the *C. jejuni* strain on broiler carcasses were also studied in

combination with activated oxygen. For this, an active oxygen generator (BIOCID MX1800-E9-IC, Oy BIOCID Ltd) was placed in the chamber (Fig. 2). To determine the reductions in bacterial counts, the carcasses were separately manually massaged for 3 min in a big Stomacher bag with sterile 100 ml of BPW. One ml of the suspension from each sample was serially diluted in 9 ml of BPW and three spread plates (mCCDA agar) were prepared from each dilution. The final log reductions were calculated as above. The tests on broiler carcasses were replicated three times for each of the three UV doses studied. All broiler carcasses used in study II were determined to be *Campylobacter*-free by the enrichment PCR method as in study I (four neck skin samples (5 g each) were pooled together to create one sample).

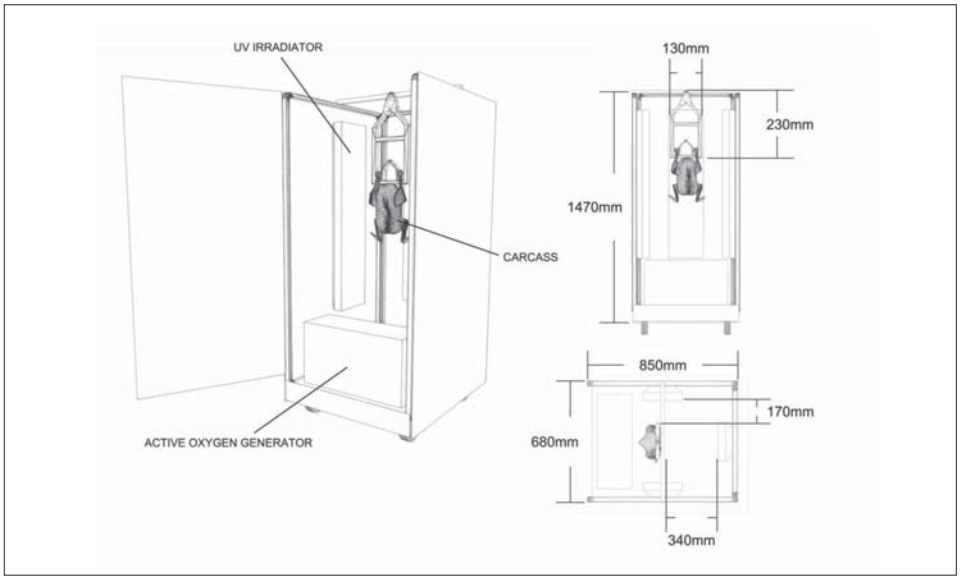

Figure 2. UV chamber with three UV irradiator units (BIOCID 110 IP55) and with an active oxygen generator (BIOCID MX1800-E9-IC) used in the studies on broiler carcasses.

4.5 SENSORY ANALYSES OF BROILER MEAT SAMPLES AFTER UV TREATMENT (II)

The sensory analyses of study II were conducted in co-operation with the Finnish Meat Research Institute (Hämeenlinna, Finland) as an outsourced service. In the analyses, refrigerated fresh broiler meat fillets with or without skin were treated with UV alone or with UV in combination with activated oxygen in the UV chamber described above. Treatment times of 10 and 100 s were used (controls not treated). Each test series contained 12 broiler meat samples either with or without skin. After the treatments, the samples were packed in batches of two in vacuum bags were vacuum degassed and stored at 4°C. In the sensory analyses, the changes in colour and sensory quality, including the visual appearance and odour of the samples, were evaluated on days 0, 2, 5, 7, 9 and 12 after the treatments. The colour of the samples was analysed using a Minolta CR-200 colorimeter (Minolta Co. Ltd., Ramsey, NJ, USA). The fatty acid composition of the samples was measured on days 0, 5, 7 and 12 after the treatments and determined using Method LA0202 (2007) with a Hewlett Packard 5890 gas chromatograph (GMI Inc., Ramsey, MN, USA).

4.6 DETERMINATION OF THE EFFECTS OF WINES AND JUICES AS POTENTIAL ANTIMICROBIAL MARINADE INGREDIENTS TO REDUCE STRAINS OF *CAMPYLOBACTER* SPECIES (III)

4.6.1 INOCULATION OF WINES AND JUICES AND DETERMINATION OF BACTERIAL COUNTS (III)

For the experiments on wines and juices in study III, four *Campylobacter* strains were used separately (RefCJ, RetCJ29, RetCC27, SlaCJ26; Table 4). The liquid types studied are described in Table 5. As a control, the survival of all the *Campylobacter* strains studied was also determined in BHI broth (LabM; pH 7.36 ± 0.03). To prepare the suspensions used for inoculations, *Campylobacter* cells from the frozen stock cultures were first plated onto mCCDA agar and incubated at 42°C for 24-48 h under microaerobic conditions obtained by CampyGen. The growth were then suspended into 5 ml of pre-warmed (42°C) BHI broths which were incubated at 42°C for 24 h under microaerobic conditions. After incubation, the optical density of the suspensions at 600 nm (OD_{600}) was adjusted to 0.1 (corresponds to log 8 cfu/ml) using 42°C BHI broth.

Table 5. Description of liquid types used in study III.

Liquid type[1]	Contents description	pH
White wine[2]	13% alcohol, sulphites	3.20 (± 0.07)
Red wine[3]	13.5% alcohol, sulphites	3.79 (± 0.05)
Grape juice[4]	Grape juice, added vitamin C, aqua, no preservatives	3.62 (± 0.07)
Tomato juice[5]	Tomato juice, glucose-fructose syrup, sugar, salt, aqua, no preservatives	4.11 (± 0.07)
Commercial poultry meat marinade	Water, turnip, rapeseed oil, honey-apple wine vinegar, maltodextrin, glucose, spices, salt (1.0%), stabilizers (E451, E450, E452), yeast extract, flavour enhancer (E621), thickeners (E412, E415), aromas, preservatives (E211, E202), acidity regulator (E330)	4.16 (± 0.03)

[1] Wines and juices were purchased from local shops, the marinade was provided by a local poultry processor.
[2] Sauvignon Blanc, Gallo Family Vineyards 2006, Modesto, CA, USA.
[3] Cabernet Sauvignon, Gallo Family Vineyards 2006.
[4] Grape nectar (72% grape nectar juice made from juice concentrates), Valio Oy, Valio, Finland.
[5] Natural tomato juice drink (100% tomato juice drink manufactured from juice concentrates), Oy Marli Ab, Turku, Finland.

To inoculate the wines and juices with high bacterial counts, 1 ml of the original suspension containing approximately 8 log cfu cells, was suspended into 9 ml of the liquid type studied to give a bacterial concentration of approximately 7 log cfu/ml. To inoculate the liquids with low bacterial counts, 1 ml from the 10^{-4} dilution tube (earlier used for counting the log cfu/ml of the original suspensions) containing approximately 4 log cfu cells was suspended into 9 ml of the liquid type studied to give a bacterial concentration of approximately 3 log cfu/ml. The inoculated samples were kept at room temperature for 3 h and then stored at 4°C. The survival of the *Campylobacter* strains in each liquid type was monitored at time-points of 0 min, < 1 min, 15 min, 30 min, 1 h, 3 h, 24 h and 48 h after inoculations. The bacterial counts were determined via the dilution plating method by using BHI broth and mCCDA agar. If the *Campylobacter* counts were below the detection limit (1 log cfu/ml), the cells were regarded as inactivated. The tests were replicated twice for each liquid type and the final results were averaged from the test replications.

4.6.2 INOCULATION AND PREPARATION OF BROILER MEAT SAMPLES AND DETERMINATION OF BACTERIAL COUNTS (III)

For the experiments on broiler meat, two *Campylobacter* strains (RefCJ and RetCC27) were used separately (Table 4). All the broiler meat samples used in study III were tested for the absence of *Campylobacter* according to ISO 10272-1:2006 (Anonymous 2006). For sample preparation, portions of 10 g of broiler meat were cut antiseptically, put into separate stomacher bags (Seward) and stored at -18°C until usage. Before inoculation, the samples were thawed for 60 min. To inoculate the meat samples with high bacterial counts, 100 µl of the original suspension containing approximately 7-8 log cfu/ml, was applied to the separate meat pieces. To inoculate the meat samples with low bacterial counts, 100 µl from the 10^{-3} dilution tube containing approximately 5 log cfu/ml was applied to the separate meat pieces. Inoculated meat pieces were kept at room temperature for 20 min to allow possible attachment and diffusion. To each inoculated meat sample, 10 ml of the corresponding liquid: white wine, red wine or Phosphate Buffered Saline (PBS, Oxoid), was added. The inoculated samples were kept at room temperature for 3 h and then stored at 4°C. The survival of the *Campylobacter* strains in each meat-liquid type was monitored at time-points of 0 min, 10 min, 15 min, 30 min, 1 h, 3 h, 24 h and 48 h after inoculations. The meat-liquid samples were homogenized in a lab blender (Stomacher 400 Circulator, Seward) before *Campylobacter* cell count and the bacterial counts were determined as above. If the *Campylobacter* counts were below the detection limit (2 log cfu/ml), the cells were regarded as inactivated. The tests were replicated three times for each meat-liquid sample and the final results were averaged from the test replications.

4.7 DETERMINATION OF THE EFFECTS OF SUBLETHAL AND LETHAL STRESSES FOR THE SURVIVAL OF *A. BUTZLERI* AND *C. JEJUNI* (IV)

Strains *A. butzleri* ATCC 49616 and *C. jejuni* NCTC 11168 were used in study IV separately (Table 4). The cells from the frozen stock cultures were plated onto Mueller-Hinton agar with Sheep Blood (MHSB; Oxoid) and incubated at 37°C for 48-72 h under microaerobic conditions obtained by CampyGen. To create the suspension used for inoculation, the growth was suspended into 5 ml of pre-warmed (37°C) BHI broth (Oxoid), which was then incubated at

37°C for 24 h under microaerobic conditions. After incubation, OD_{600} was adjusted to 0.1 using 37°C BHI broth. To determine sublethal stress adaptation conditions for the *A. butzleri* and *C. jejuni* strains used in study IV, their survival was studied individually at 48°C (heat stress), at 10°C (cold stress) and at pH 5.0 at 37°C (mild acid stress). In addition, to determine the lethal acid stress conditions, their survival was studied at pH 4.0 at 37°C. The conditions were chosen based on earlier survival studies conducted on *A. butzleri* and *C. jejuni* (Murphy et al. 2006, Cervenka 2007, Van Driessche, Houf 2008, Kjeldgaard et al. 2009, Jackson et al. 2009) and on own preliminary experiments (data not shown). For inoculation, 1 ml of the suspension containing about log 8 cfu cells was suspended into 9 ml of BHI broth resulting in an original bacterial concentration of approximately 7 log cfu/ml. Before inoculation with the bacterial suspension, BHI broth tubes had been adjusted to the different study temperatures (48°C, 10°C or 37°C) using incubators and to pH 5.0 or pH 4.0 with filter-sterilized (SLHA033SS, MILLEX-HA Filter Unit 0.45µm, MILLIPORE, Carrigtwohill, Ireland) 15% (w/v) Tartaric acid (Sigma-Aldrich). In the cross-protection part of study IV, cells were first inoculated as described above and adapted individually to heat stress (2 h at 48°C), cold stress (24 h at 10°C) or mild acid stress (4 h at pH 5.0). After adaptations, survival of the bacterial strains was studied individually under mild or lethal acid stress conditions by inoculating 1 ml of the adapted cells into 9 ml of BHI broth with a pH of 5.0 or 4.0 (at 37°C), resulting in an original bacterial concentration of about log 6 cfu/ml. The bacterial counts in study IV were determined in each condition at time-points of 0 h, 1 h (at pH 4.0 only), 2 h, 4 h and 24 h after inoculation via the dilution plating method by using BHI broth and MHSB agar. If the bacterial counts were below the detection limit (1 log cfu/ml), the cells were interpreted as inactivated. In order to assure the reproducibility of the findings, the experiments were replicated at least four times for each study condition. More replicates were performed in the condition under which cross-protection was found likely, i.e., heat stress adapted *A. butzleri* (Table 9 and Fig. 4). Survival at pH 4.0 at 1 h for non-adapted *A. butzleri* was studied altogether 12 times and 16 times for heat stress adapted *A. butzleri*.

4.8 STATISTICAL ANALYSIS (I-IV)

For data management and calculations in study I, Microsoft Excel 97 SR 2 and SAS Systems vers. 8 (Cary, NC, USA) were used. The level of agreement according to precision was expressed as the kappa statistic, defined as the proportion of potential agreement beyond chance exhibited by two tests. Diagnostic specificity was calculated as: $d/(b + d)$ where d is the number of samples negative both by PCR and by culture and b is the number of samples positive by PCR, but negative by culture. The level of agreement between two tests was calculated as: $(a + d)/n$, where a is the number of samples positive both by PCR and by culture, d is the number of samples negative by both methods and n is the total number of samples under examination.

In study II, the statistical differences between the effects of the three doses of UV irradiation on broiler meat and skin were determined by analyses of variance, where the initial concentration was used as a covariate. The statistical differences between the effects of the three doses of UV alone or of UV in combination with activated oxygen on broiler carcasses were determined by analyses of variance. All analyses were performed by means of SPSS 14.0 for Windows.

In study III, the data from each time-point and the statistical differences between the main effects of the bacterial strains and the liquid types were all determined separately. Because data were not normally distributed, the significances were determined by the nonparametric

Kruskal-Wallis Test. Analyses were performed by means of SPSS 16.0 for Windows statistical package.

In study IV, differences in the medians of viable bacteria counts of heat stress adapted *A. butzleri* strain and non-adapted *A. butzleri* strain at pH 4.0 were analysed using Wilcoxon rank sum test (R, Vienna, Austria). More precisely, the one-tailed version of the test was used to examine whether more bacteria that were viable resulted after heat stress adaptation than without adaptation at pH 4.0. The results of studies I-IV were considered to be statistically significant when $P < 0.05$.

5. RESULTS

5.1 *CAMPYLOBACTER* PREVALENCE IN FINNISH RETAIL POULTRY MEAT PRODUCTS (I)

The isolation rates of *Campylobacter* in different types of poultry meat products are shown in Table 6. Using either the conventional culture or PCR method, a total of 25 (12.9%) of the 194 samples investigated were *Campylobacter* positive. Out of 136 broiler and 56 turkey meat products, 20 (14.7%) and four (7.1%) samples respectively, were *Campylobacter* positive. One of the two mixed broiler and turkey meat samples tested positive for *Campylobacter*. *Campylobacter* was detected in 19.0% of the non-marinated and in 10.3% of the marinated poultry meat products. The occurrence of *Campylobacter* was 9.4% in poultry meat slices and barbecue sticks, 4.8% in breast fillets and 30.4% in products with skin and bone. *Campylobacter* was not detected in any of the 22 poultry products with meat of foreign origin.

Table 6. Types of Finnish retail poultry meat products and *Campylobacter* positive samples.

Product type	No. of samples positive[1]/ No. of samples tested			
	Slices and barbecue sticks	Breast fillets and fillet steaks	Breasts, legs, drumsticks and wings including bones and skin	All
Non-marinated[2]	0/13	2/19	9/26	11/58
Marinated	8/72	1/44	5/20	14/136
Total samples	8/85	3/63	14/46	25/194

[1] No. of samples tested positive by microbiological method and/or PCR method.

[2] Natural, lightly salted or spiced products.

Between June-September, a peak in the *Campylobacter* prevalence was observed (data not shown). In August, the peak was highest, with 28.9% prevalence in the 45 samples investigated. Between January-May, *Campylobacter* was detected in only one of 50 samples studied.

5.2 COMPARISON OF THE CULTURE AND PCR METHOD (I)

Eighteen of 194 samples were positive using the conventional culturing method and 24 were positive using the PCR method for *Campylobacter*. One sample gave a positive result by culture, but was negative by PCR. Seven samples were positive by PCR, but culture negative. From five of these samples, approximately 400 bp of the PCR product was sequenced and all sequences were 99% or 100% equal to *C. jejuni*. The results of the culture and PCR were concordant in 186 samples, representing 96.4% of the samples. The diagnostic specificity for the comparison of the PCR to culture by selective enrichment was 0.96 with a level of agreement of 0.96. The detection

limit of both enrichment culture and enrichment PCR was less than 1 cfu/ml of sample rinse, while the detection limit of direct culture was 70 cfu/ml. For the direct PCR detection, the limit was 700 cfu/ml of sample rinse. When the specificity of the C412F-16S rRNA-campR2 primer set was tested against a panel of *Campylobacter* and non-*Campylobacter* DNA templates (Table 3), the PCR assay detected *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. helveticus*, and *C. hyointestinalis*, but none of the other *Campylobacter* species tested. No signal was observed for any of the *Arcobacter*, *Helicobacter*, or other non-*Campylobacter* species tested. A tendency was seen that this primer set captured more of the samples that were culture negative and negative with the MD16S1 and MD16S2 primers (data not shown). However, the differences were not statistically significant.

5.3 EFFECTS OF UV IRRADIATION TO REDUCE *C. JEJUNI* E1 1347 ON AGAR PLATES AND ON BROILER MEAT, SKIN AND CARCASSES (II)

In study II, in the tests conducted on agar plates, at least a 6.3 log cycle reduction in *C. jejuni* E1 1347 counts (99.9% inactivated) was observed with all the UV irradiation doses studied (data not shown). The maximum reduction in *C. jejuni* E1 1347 achieved with UV irradiation was 0.7 log cycles on broiler meat, 0.8 log cycles on broiler skin and 0.5 log cycles on broiler carcasses (Table 7). On broiler meat, there were statistically significant differences between the effects of the lowest and highest doses studied ($P = 0.030$), but on broiler skin and carcasses no such differences were found. On broiler meat, the effects of the three doses depended significantly on the initial concentration of *C. jejuni* E1 1347 inoculated ($P = 0.029$), which was variable throughout the test replications. The lower the initial concentration, the better the reducing capacity of UV irradiation was. On broiler skin and carcasses, the effects of UV irradiation did not significantly depend on the initial concentration of *C. jejuni* E1 1347, although it varied. When using UV irradiation in combination with activated oxygen on broiler carcasses, the effects in reducing *C. jejuni* E1 1347 counts did not increase (Table 7).

Table 7. The log counts (cfu/ml) of *C. jejuni* E1 1347 (mean ± SD) before and after treatment with UV irradiation on the surfaces of broiler meat (n = 5), broiler skin (n = 5) and broiler carcasses (n = 3).

Surface type	Low UV dose[1]		Medium UV dose[2]		High UV dose[3]	
	Initial counts	Final counts	Initial counts	Final counts	Initial counts	Final counts
Broiler meat	7.8 ± 0.2	7.3 ± 0.4	7.7 ± 0.4	7.0 ± 0.6	8.0 ± 0.4	7.3 ± 0.5
Broiler skin	7.8 ± 0.3	7.0 ± 0.1	8.1 ± 0.1	7.3 ± 0.2	7.9 ± 0.1	7.1 ± 0.1
Broiler carcasses	7.3 ± 0.8	7.0 ± 0.8	7.3 ± 0.8	6.9 ± 0.9	7.3 ± 0.8	6.8 ± 0.8
Broiler carcasses[4]	8.0 ± 0.3	7.7 ± 0.3	8.0 ± 0.3	7.7 ± 0.2	8.0 ± 0.3	7.5 ± 0.4

[1] 9.4 mWs/cm^2 on broiler meat and skin and 10.8 mWs/cm^2 on broiler carcasses.

[2] 18.8 mWs/cm^2 on broiler meat and skin, and 18.0 mWs/cm^2 on broiler carcasses.

[3] 32.9 mWs/cm^2 on broiler meat and skin, and 32.4 mWs/cm^2 on broiler carcasses.

[4] Effects of UV irradiation studied in combination with activated oxygen.

No significant differences were found in the colour, sensory quality or in the fatty acid compositions of the control broiler meat samples and the broiler meat samples treated with UV or with UV in combination with activated oxygen although minor differences in colour values were detected in a few individual samples (data not shown).

5.4 EFFECTS OF WINES AND JUICES AS ANTIMICROBIAL MARINADE INGREDIENTS TO REDUCE STRAINS OF *CAMPYLOBACTER* SPECIES (III)

Table 8 shows that of all the liquids used in study III, white wine had the strongest antibacterial effect on the survival of all four different *Campylobacter* strains studied. High counts of all strains were inactivated within 15 min and the low inoculum levels of RetCC27 and RefCJ were inactivated to undetectable numbers within < 1 min in white wine. In red wine, high counts of all *Campylobacter* strains were reduced to low counts within 30 min and inactivated within 1 h. Both grape and tomato juice were less bactericidal than the wines. In grape and tomato juice, the high inoculum levels of SlaCJ26, RetCJ29 and RefCJ were still detected after 48 h exposure. The low counts of the strains RefCJ and RetCJ29 in grape juice and of the strain RetCJ29 in tomato juice were still detected after 48 h exposure. In the commercial marinade, the high counts of most of the *Campylobacter* strains were inactivated within 48 h exposure and all the low counts were inactivated to undetectable numbers within 3 h (Table 8). In the BHI broth control solution, the counts of *Campylobacter* did not change significantly within 48 h (data not shown).

Figure 3 shows that both red and white wines reduced the counts of both *Campylobacter* strains inoculated on broiler meat moderately by approximately 1 log cfu/ml within 48 h. These *Campylobacter* strains were still detectable in fairly high numbers after 48 h exposure to the wines when inoculated on meat. The statistical differences between the effects of the different liquid types at each time-point were examined using the data acquired from the *Campylobacter* strains with both high and low inoculum levels. There were statistically significant differences ($P \leq 0.001$) in the survival of *Campylobacter* strains in the different liquid types at every time-point, except at the 48 h time-point with the low inoculum levels. There were no statistically significant differences between the different *Campylobacter* strains in each of the liquid types studied with either of the inoculum levels or between the counts of *Campylobacter* in meat-wine samples to meat-PBS samples.

Table 8. Counts[1] (log cfu/ml) of *Campylobacter* strains during various exposures to the liquids studied.

Liquid type	Exposure time	*Campylobacter* strain							
		RefCJ		RetCJ29		RetCC27		SlaCJ26	
		High	Low	High	Low	High	Low	High	Low
Red wine	0 min	7.2 ± 0.1	3.2 ± 0.1	7.0 ± 0.2	3.0 ± 0.2	7.4 ± 0.0	3.4 ± 0.0	7.1 ± 0.0	3.1 ± 0.0
	< 1 min	6.8 ± 0.5	2.6 ± 0.1	6.2 ± 0.4	2.3 ± 0.5	6.4 ± 0.6	2.5 ± 0.3	6.2 ± 0.3	2.7 ± 0.3
	15 min	< 1.0	< 1.0	3.0 ± 4.3	1.0 ± 1.4	1.0 ± 1.4	< 1.0	< 1.0	< 1.0
	30 min	< 1.0	< 1.0	1.4 ± 2.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	1 h	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
White wine	0 min	7.1 ± 0.3	3.1 ± 0.3	6.8 ± 0.2	2.8 ± 0.2	7.7 ± 0.0	3.7 ± 0.0	6.6 ± 0.4	2.6 ± 0.4
	< 1 min	2.2 ± 3.1	< 1.0	4.8 ± 1.6	1.1 ± 1.6	1.4 ± 1.9	< 1.0	4.3 ± 2.9	1.2 ± 1.6
	15 min	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
Grape juice	0 min	7.4 ± 0.1	3.4 ± 0.1	7.2 ± 0.0	3.2 ± 0.0	7.5 ± 0.3	3.5 ± 0.3	7.2 ± 0.1	3.2 ± 0.1
	24 h	6.4 ± 0.3	2.5 ± 0.1	6.5 ± 0.2	2.8 ± 0.8	1.2 ± 1.8	< 1.0	6.1 ± 0.1	1.0 ± 1.4
	48 h	5.6 ± 0.0	1.0 ± 1.4	5.2 ± 0.6	1.3 ± 1.8	< 1.0	< 1.0	5.1 ± 0.3	< 1.0
Tomato juice	0 min	7.2 ± 0.1	3.2 ± 0.1	7.1 ± 0.1	3.1 ± 0.1	7.6 ± 0.3	3.6 ± 0.3	7.2 ± 0.2	3.2 ± 0.2
	24 h	6.7 ± 0.4	2.0 ± 0.0	6.8 ± 0.3	2.5 ± 0.4	1.2 ± 1.8	< 1.0	6.4 ± 0.1	1.5 ± 2.1
	48 h	5.9 ± 0.4	< 1.0	6.2 ± 0.8	1.1 ± 1.6	< 1.0	< 1.0	4.4 ± 0.1	< 1.0
Marinade	0 min	7.2 ± 0.0	3.2 ± 0.0	7.1 ± 0.0	3.1 ± 0.0	7.7 ± 0.0	3.7 ± 0.0	7.5 ± 0.0	3.5 ± 0.0
	1 h	6.7 ± 0.1	3.0 ± 0.3	6.8 ± 0.0	2.8 ± 0.1	7.3 ± 0.5	1.6 ± 2.2	6.8 ± 0.2	2.4 ± 0.6
	3 h	5.5 ± 0.3	< 1.0	5.1 ± 0.5	< 1.0	5.0 ± 1.4	< 1.0	4.1 ± 0.4	< 1.0
	24 h	3.7 ± 0.4	< 1.0	2.0 ± 0.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	48 h	1.0 ± 1.4	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

[1] Mean (n = 2) ± standard deviation. Detection limit was 1 log cfu/ml

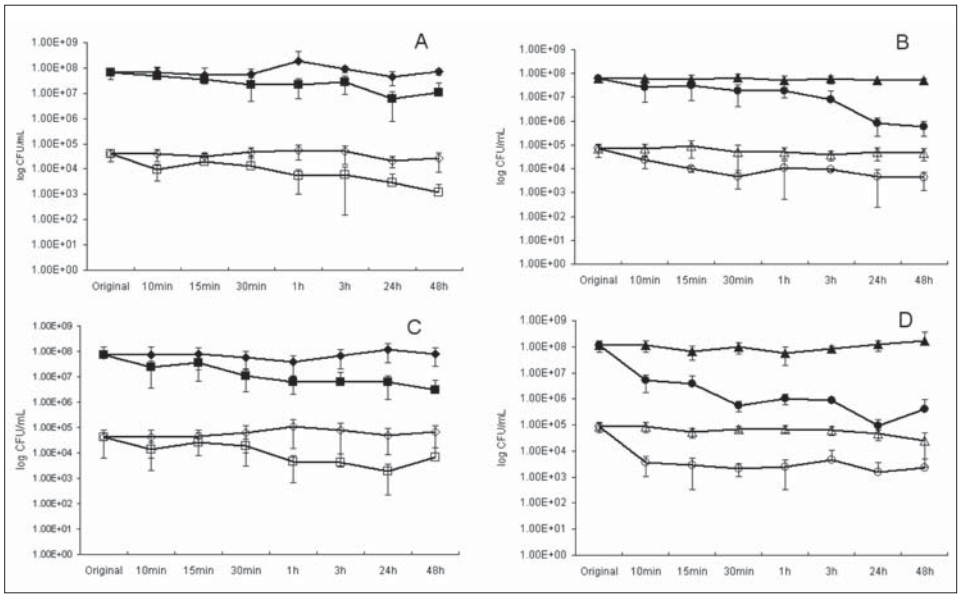

Figure 3. Survival of high and low counts of the *Campylobacter* strains RefCJ (A and B) and RetCC27 (C and D) in red wine marinated meat (A and C) and white wine marinated meat (B and D) determined at different time-points. ♦/◊ and ▲/Δ are RefCJ/RetCC27 counts in meat/PBS, ■/□ are RefCJ/RetCC27 counts in meat/red wine, ●/○ are RefCJ/RetCC27 counts in meat/white wine. Error bars represent the standard deviation. The detection limit was 2 log cfu/ml.

5.5 SURVIVAL OF *C. JEJUNI* NCTC 11168 AND *A. BUTZLERI* ATCC 49616 IN STRESS CONDITIONS (IV)

In study IV, the counts of *A. butzleri* ATCC 49616 and *C. jejuni* NCTC 11168 decreased to undetectable numbers within 24 h at 48°C, but after 2 h, counts of both bacteria had decreased only moderately. A moderate decrease in the bacterial counts was also seen after 24 h at 10°C. Thus, sublethal heat and cold stress adaptations of the bacteria were determined to be conducted by incubating the cells for 2 h at 48°C or for 24 h at 10°C, respectively. The mild acid stress adaptations were chosen to be performed by incubating the bacteria for 4 h at pH 5.0, because their counts had moderately decreased after that. Incubation at pH 4.0 was chosen to represent the lethal acid stress condition, because there *A. butzleri* ATCC 49616 was inactivated to undetectable counts within 1 h and *C. jejuni* NCTC 11168 within 24 h (Table 9).

In the cross-protection part of study IV, heat or cold stress adaptations did not improve the survival of the bacteria at pH 5.0. This was also confirmed by the statistical analysis that did not find evidence for better survival after adaptation than without adaptation. For *A. butzleri* ATCC 49616, heat stress adaptation improved survival at the 1 h time-point at pH 4.0 (Table 9 and Fig. 4). The heat stress adapted *A. butzleri* ATCC 49616 cells were significantly ($P < 0.01$) more resistant to subsequent lethal acid stress than non-adapted cells at the 1 h time-point. Non-adapted *A. butzleri* ATCC 49616 cells did not survive at pH 4.0 in any of the study replications, whereas viable heat adapted cells were counted up to 10^4 cfu/ml at the 1 h time-point (Fig. 4). All non-adapted *A. butzleri* ATCC 49616 cells were inactivated to undetectable counts within 1 h at pH 4.0 (in 12 out of 12 study replications), whereas heat stress adapted cells were still detectable

after 1 h at pH 4.0 in seven cases of the study replications (n=16; Fig. 4). Under any other condition studied, the statistical test showed no evidence for cross-protection. At later time-points at pH 4.0, no significant differences between the survival of heat stress adapted and non-adapted *A. butzleri* ATCC 49616 were observed. For *C. jejuni* NCTC 11168, heat stress adaptation did not improve survival of the bacteria at pH 4.0. For both bacteria, the mild acid or cold stress adaptations did not improve the survival of the bacteria at pH 4.0. Like non-adapted *A. butzleri* ATCC 49616 cells, the mild acid or cold stress adapted cells were inactivated to undetectable counts within 1 h at pH 4.0. For *C. jejuni* NCTC 11168, the cold or acid adaptations actually decreased the survival times in pH 4.0, because the adapted cells were inactivated to undetectable counts within 4 h (non-adapted within 24 h; Table 9).

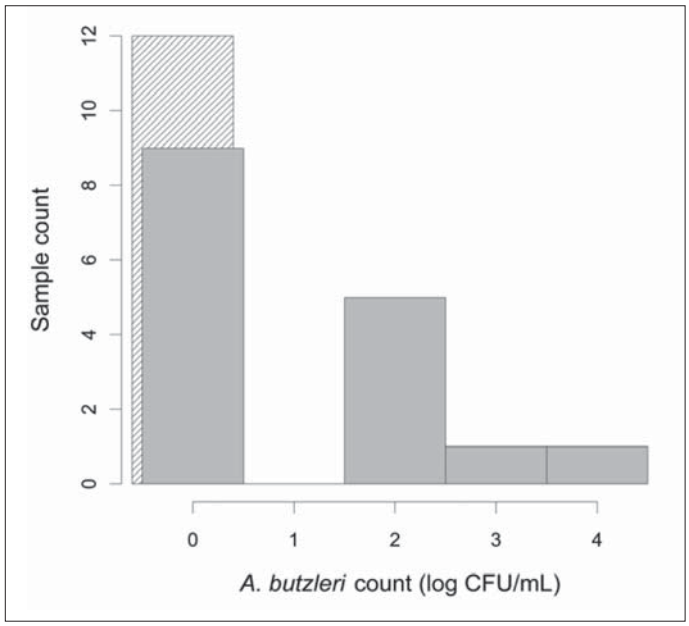

Figure 4. Distributions of viable *A. butzleri* ATCC 49616 cell counts at pH 4.0 at the 1 h time-point without adaptation (column with stripes) and after heat stress adaptation (columns without stripes).

Table 9. Counts[a] (log cfu/ml) of *A. butzleri* ATCC 49616 and *C. jejuni* NCTC 11168 during exposure at 48°C, 10°C, pH 5.0 and pH 4.0 without adaptation and after heat, acid or cold stress adaptations. The bold values indicate the comparison where cross-protection was observed. Detection limit was 1 log cfu/ml.

Condition	Exposure time (h)	*C. jejuni*				*A. butzleri*			
		Non-adapted	Heat-adapted	Cold-adapted	Acid-adapted	Non-adapted	Heat-adapted	Cold-adapted	Acid-adapted
48°C	0	7.6 ± 0.1	nd[b]	nd	nd	7.7 ± 0.2	nd	nd	nd
	2	7.3 ± 0.3	nd	nd	nd	6.7 ± 1.0	nd	nd	nd
	4	7.2 ± 0.4	nd	nd	nd	5.4 ± 1.2	nd	nd	nd
	24	< 1.0	nd	nd	nd	< 1.0	nd	nd	nd
10°C	0	7.6 ± 0.2	nd	nd	nd	7.9 ± 0.2	nd	nd	nd
	24	7.5 ± 0.3	nd	nd	nd	7.0 ± 0.1	nd	nd	nd
pH 5.0	0	7.5 ± 0.3	6.6 ± 0.0	6.6 ± 0.2	nd	7.6 ± 0.1	6.4 ± 0.1	6.0 ± 0.1	nd
	2	7.4 ± 0.2	6.4 ± 0.1	6.4 ± 0.3	nd	7.7 ± 0.1	6.2 ± 0.1	5.6 ± 0.1	nd
	4	7.3 ± 0.3	6.3 ± 0.1	6.5 ± 0.2	nd	7.5 ± 0.2	6.1 ± 0.1	5.4 ± 0.1	nd
	24	7.1 ± 0.3	3.4 ± 2.3	4.6 ± 0.2	nd	5.6 ± 0.1	< 1.0	< 1.0	nd
pH 4.0	0	7.7 ± 0.1	6.5 ± 0.0	6.1 ± 0.3	6.4 ± 0.1	7.6 ± 0.2[c]	6.1 ± 0.2[d]	6.4 ± 0.4	6.4 ± 0.1
	1	7.5 ± 0.1	6.3 ± 0.2	5.7 ± 0.2	5.9 ± 0.9	**< 1.0[c, e]**	**1.0 ± 1.3[d, e]**	< 1.0	< 1.0
	2	6.9 ± 0.1	5.1 ± 1.4	3.9 ± 0.8	2.9 ± 1.9	< 1.0[c]	< 1.0[d]	< 1.0	< 1.0
	4	3.7 ± 0.8	0.6 ± 1.2	< 1.0	< 1.0	< 1.0[c]	< 1.0[d]	< 1.0	< 1.0
	24	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0[c]	< 1.0[d]	< 1.0	< 1.0

[a] Mean (n = 4) ± standard deviation.
[b] Not determined.
[c] Mean (n = 12) ± standard deviation.
[d] Mean (n = 16) ± standard deviation.
[e] P < 0.01

6. DISCUSSION

6.1 SURVIVAL OF *CAMPYLOBACTER* IN RETAIL POULTRY MEAT

The exact sources of human *Campylobacter* infections in Finland are not clear at the moment. A high percentage of the infections are considered to originate from travel abroad but in many of the cases the source is unknown. However, in the summer period the proportion of *Campylobacter* infections acquired in Finland is considerable and it has been estimated that almost one in three of these infections is associated with broilers (THL 2012, Zoonosis Centre 2012). In study I the *Campylobacter*-contamination status of both marinated and non-marinated broiler and turkey meat products in Finland was indicated to be rather low, which is consistent with earlier studies carried out in Finland (Hänninen et al. 2000, EFSA 2006). Compared with many other countries the prevalence of *Campylobacter* in Finnish poultry products seems to be relatively low (Suzuki, Yamamoto 2009). However, the results can not be directly compared because of the different detection methods used. The low occurrence of *Campylobacter* in Finnish retail poultry meat products probably reflects the low *Campylobacter* prevalence observed in the broiler slaughter batches in Finland, which, since 2004, has been 6.5% in all broiler flocks slaughtered between June-October on average (Zoonosis Centre 2012). Prevention of *Campylobacter* at the farm level by a high level of biosecurity control and hygiene are the probable reasons for the low prevalence in Finland (Perko-Mäkelä 2011). The higher prevalence indicated in the meat products compared with the prevalence in broiler flocks could however be speculated to be due to the possibility of negative flocks becoming contaminated at the slaughter process (Perko-Mäkelä et al. 2009). Still, it has been reported that *Campylobacter* contamination of poultry can occur at all stages of the production chain (Perko-Mäkelä 2011). Because *Campylobacter* were recovered from the retail poultry meat products used in study I, the results indicate that *Campylobacter* might be transmitted into the broiler meat production chain also in Finland during processing.

As expected, a seasonal variation observed in *Campylobacter* incidence in broiler flocks in Finland was also detected in the retail poultry meat products of study I. Boysen, Vigre & Rosenquist (2011) found that season had a significant effect on the occurrence of *Campylobacter* in fresh, chilled, Danish broiler meat at the retail level, even though flock prevalence was also found to be a strong predictor for *Campylobacter* prevalence. The explanation for the direct effect of season was not, however, linked directly to broiler flock prevalence, but assumed to be found in the production line before the retail level (Boysen et al. 2011). In Finland, the exact reason for the seasonal variation remains unknown, but several factors are probably important. Mean temperature and rainfall, for example, and the higher and earlier *Campylobacter* infection pressure from outside the broiler house might affect the flock prevalence during the warmer months, whereas cold winters on the other hand might decrease the environmental load of *Campylobacter* (Hald et al. 2004, Rushton et al. 2009, Jore et al. 2010).

In study I, *Campylobacter* were detected also in marinated poultry meat products. Regarding this, it is important to be aware that most of the retail poultry meat sold today in Finland is marinated (Björkroth 2005). To exclude the effects of season on the *Campylobacter* prevalence in marinated poultry meat products, these products were included in the samples collected from the retail shops each month during the sampling period. *Campylobacter* has been detected in marinated poultry products in other studies, too (Atanossava et al. 2007, Lindmark et al. 2009, Baumgartner, Felleisen 2011). Baumgartner and Felleisen (2011), however, indicated low *Campylobacter* contamination rates in marinated broiler meat products. They speculated this to be because of meat processing, where *Campylobacter* were exposed to oxygen for longer times or where spices or acid components of marinades contributed to the reduction in *Campylobacter* counts. In contrast, Perko-Mäkelä et al. (2000) found no difference in the survival of *Campylobacter* between marinated and non-marinated broiler meat. Also in study I, *Campylobacter* was detected in both marinated and non-marinated poultry meat products, indicating that marinating meat might not affect the survival of *Campylobacter*.

The reason why *Campylobacter* was not detected in any of the poultry products with meat of foreign origin in study I could be that foreign meat is frozen when imported to Finland. Baumgartner and Felleisen (2011), for example, reported that the proportion of broiler meat samples contaminated with *Campylobacter* was significantly lower for deep frozen than for refrigerated material in their study. In study I, a relatively high *Campylobacter* prevalence was indicated in the poultry meat products with skin and bone, compared to the overall prevalence in the products. Correspondingly, studies have shown that the levels of *Campylobacter* surface contamination in broiler meat products are far larger than those of deep tissue contamination (Scherer et al. 2006, Luber, Bartelt 2007). Interestingly, Baumgartner and Felleisen (2011) reported that a combination of skinning and deep-freezing seemed to be highly effective to reduce the *Campylobacter* load on broiler meat.

Studies showing that the *Campylobacter* levels are high especially on the surfaces of contaminated broiler meat products suggest that cross-contamination from the meat surface is the risk for gaining the infection, rather than eating broiler that is perceived to be undercooked (Scherer et al. 2006, Luber, Bartelt 2007). Consumers are still responsible for the use of proper food handling techniques in domestic kitchens. In general, *Campylobacter* and also *Arcobacter* are rather easily inactivated by heat treatments. For example, study IV showed that *C. jejuni* NCTC 11168 and *A. butzleri* ATCC 49616 were inactivated within 24 h at 48°C in broth, which corresponds to studies of Blankenship and Craven (1982), ICMSF (1996), Hilton et al. (2001), D'Sa and Harrison (2005), Habib et al. (2010) and Al Sakkaf and Jones (2012). It has been reported that approximately 25% of domestic refrigerators may have temperatures exceeding 10°C (Laguerre et al. 2002). In study IV, only a moderate decrease in the *C. jejuni* NCTC 11168 and *A. butzleri* ATCC 49616 counts was seen after 24 h incubation at 10°C in broth. Comparative results have been published by Phillips and Duggan (2002) and by Murphy et al. (2006). Kjeldgaard et al. (2009) reported growth of *A. butzleri* at 10°C in CMJ. Chilling and freezing have been shown to maintain a small number of live *Campylobacter* in broiler meat, but to still reduce their counts rapidly (Solow et al. 2003, Bhaduri, Cottrell 2004, Georgsson et al. 2006, Pintar et al. 2007, El-Shibiny et al. 2009, Oyarzabal et al. 2010, Sampers et al. 2010). Thus, it is highly recommended to store fresh broiler meat at temperatures lower than 10°C. The maintenance of the refrigeration chain during transport should also be ensured.

The popularity of consumption of especially of marinated broiler and turkey meat has been increasing in Finland during recent years (http://www.siipi.net). In conclusion, study I indi-

cates that such products can be contaminated with *Campylobacter* with a low prevalence in Finland. In the summer, however, there is a high seasonal peak in the prevalence. *Campylobacter* contamination might lead to human infection if the food is not handled properly by the consumer. The *Campylobacter* detection methods used in study I provided only information on the absence/presence of *Campylobacter* in the retail poultry meat products studied. Given the fact that the numbers of *Campylobacter* cells are highly important for the risk of human illness, quantitative methods should be used for the detection of the occurrence of *Campylobacter* in Finnish poultry meat products.

6.2 PCR ASSAY FOR THE DETECTION OF *CAMPYLOBACTER* IN MARINATED POULTRY MEAT PRODUCTS

The popularity and the variety of marinated poultry meat products in Finland are high, and thus reliable methods for the detection of *Campylobacter* in these products are of interest to laboratories in routine work and research. In study I, a PCR assay was compared with the conventional culture method for the detection of *Campylobacter* in the poultry meat products studied. Good correlation in the comparison between the PCR and the cultural detection by selective enrichment was found. In one sample, the result using culture method was positive, but in PCR the result was negative. This false-negative result may be explained by the fact that the size of the subsample used for the culture method was larger than in the PCR method. Seven samples gave a positive result with PCR after enrichment, whereas the culture result was negative. However, sequencing revealed five of the PCR products to be *C. jejuni*. PCR methods detect also dead or non-viable *Campylobacter*. This might have influenced the results, especially in samples which have been frozen or subjected to low pH marinades. The samples, however, had been taken from both marinated and non-marinated products (with or without skin), and none of the samples had been frozen. Another reason for the culture negative/PCR positive results might be the abundant growth of the background flora observed on mCCDA plates in 5% of all samples, which in some cases made *Campylobacter* detection impossible.

Susceptibility to inhibitory substances, which can be found in high levels in foods, is a great disadvantage of PCR. Lilja and Hänninen (2001), for example, reported problems in the preparation of marinated broiler samples prior to PCR analysis. In study I, an original DNA isolation kit protocol was modified to be performed especially on marinated poultry meat products. For this, a pre-centrifugation step was performed on the samples in order to exclude most of the lipids and fat from the marinade and the poultry meat skin. As DNA isolation was performed manually with a DNA isolation kit for automated DNA isolation, further optimization compared to the manufacturer's instructions was necessary to make the manual DNA isolation as sensitive as the automated isolation. The most important step was found to be vigorous vortexing of the samples in lysis buffer. To optimize DNA isolation from marinated poultry products, one possibility could be to add fat digesting enzymes to the bacterial pellet just prior to DNA isolation.

In study I, to control the PCR reaction in the different samples studied, an internal control PCR was run simultaneously with the target DNA. In both PCR reactions, performed on DNA isolated directly from the samples and on DNA isolated from the enrichment media, the internal control gave a band of the same intensity showing no evidence of inhibition of the PCR reaction. However, the detection limit of the direct PCR was about 700 cfu/ml. This is high compared to other direct PCR assays for *Campylobacter*. Lund et al. (2003), for example, reported a detec-

tion limit of approximately 40 cfu/ml in faecal material. As inhibition of the PCR reaction does not seem to be the problem, it may also be possible that *Campylobacter* are preferably located in the fatty part of the sample and so many bacteria might be lost since this part is removed before DNA isolation. On the other hand, the fat and or protein still present in a sample after pre-treatment could interfere with DNA isolation. As the detection limit of the present direct PCR was too high compared to the normally quite low amount of *Campylobacter* in food and retail poultry samples, it was necessary to perform a combination of enrichment and PCR assay.

Traditional conventional culture methods include enrichment and plating steps followed by isolation of the bacterium and biochemical identification of the isolate. In conclusion, the PCR method used shortens time compared to these methods even though enrichment of the samples was necessary and could therefore be used for detection of *Campylobacter* in poultry meat products. The optimized DNA isolation method could be used in studies concerning marinated poultry meat products.

6.3 THE EFFECTS OF VARIOUS INGREDIENTS AND CONDITIONS IN THE REDUCTION OF STRAINS OF *CAMPYLOBACTER* SPECIES IN BROILER MEAT

Campylobacter constitutes a major public health problem worldwide and many efforts have been directed against finding appropriate intervention methods to control *Campylobacter* during all the steps of the broiler meat production chain. Despite the efforts, it has been shown that elevated levels of *Campylobacter* can be recovered from broiler carcasses at the slaughterhouse and transmitted into the food chain during further processing (EFSA 2010a). Rather than reducing *Campylobacter* prevalence in positive broiler flocks, it is thought the most effective way to control *Campylobacter* in broiler is to reduce their levels on carcasses after evisceration (Hermans et al. 2011). One potential decontamination technique could be to use UV irradiation to reduce the counts of *Campylobacter* in the contaminated broiler carcasses at the slaughterhouse.

In study II, it was shown that UV irradiation was very effective in reducing *C. jejuni* E1 1347 counts on agar plates. Other authors also found UV to be effective in reducing other bacteria such as *E. coli* or *Salmonella* spp. on the surfaces of agar plates (Stermer et al. 1987, Sumner et al. 1996, Wong et al. 1998, Kim et al. 2002, Yaun et al. 2004). UV treatment has been effective in reducing *C. jejuni* in liquid samples, too (Butler et al. 1987, Haughton et al. 2011). It seems that agar surfaces and liquids have properties that do not reduce the penetration ability of UV light. This could explain why UV is so effective on smooth surfaces (Lyon et al. 2007). However, on broiler meat and skin, UV irradiation was less effective in eliminating *C. jejuni* E1 1347 than on agar plates. The reductions achieved with UV irradiation in many earlier studies with other bacteria on meat have been more effective compared to this study (Stermer et al. 1987, Wong et al. 1998, Kim et al. 2002). Lyon et al. (2007), for example, showed reductions of about 2 log in *L. monocytogenes* on broiler breast fillets after UV treatment. However, Haughton et al. (2011) obtained a reduction of 0.76 log cfu/g in *C. jejuni* counts on raw broiler fillets following UV treatment of up to 0.192 joules per square centimetre (J/cm^2), which concurred with the findings of this study. Chun et al. (2010) reported a 1.26 log cfu/g reduction of *C. jejuni* on skinless broiler breast following UV irradiation of 0.50 J/cm^2, which is a much greater dose compared to that used in study II (32.9 mW/s/cm^2 = 0.0329 J/cm^2) and to the study of Haughton et al. (2011). This suggests that increasing the UV dose in study II might have improved the decontamination

potential of this technology for broiler meat. In study II, UV irradiation had no deleterious effects on the sensory quality of broiler meat which has been reported by Stermer et al. (1987), Wallner-Pendleton et al. (1994), Lyon et al. (2007), Chun et al. (2010), and Haughton et al. (2011), too. On broiler carcasses, UV irradiation was even less effective in reducing *C. jejuni* E1 1347. A 61% reduction in *S.* Typhimurium counts on poultry carcasses was observed by Wallner-Pendleton et al. (1994), which is almost similar to the findings of our study with *C. jejuni* E1 1347, where the maximum % reduction on broiler carcasses was 62.7%. Using UV irradiation in combination with activated oxygen did not significantly increase the reducing effects of UV radiation on broiler carcasses. This could be due to the short treatment times used in this study (only up to 18 s). Overall, the modest reductions gained in study II could be because the cut edges in the meat and the uneven shape of carcasses probably created shadows and interfered with the penetration of UV radiation as observed by Lyon et al. (2007). Lower doses and shorter treatment times used in the present study, and the testing of other bacteria than *C. jejuni* in the other studies, might also explain the differences compared to earlier studies. In addition, the initial concentration of the present *Campylobacter* inoculants was higher than could be expected to occur on naturally contaminated broiler carcasses at the slaughterhouse (EFSA 2010a). Furthermore, the effects of UV irradiation on *C. jejuni* strains of different origin and at different growth stages might differ greatly (Yaun et al. 2003, Haughton et al. 2011). Study II was conducted using just one *C. jejuni* strain. In future, in order to gain valid results for *Campylobacter* in general, more strains of *Campylobacter* species should be included in decontamination studies, for example, as a mixture.

Another potential technique to control *Campylobacter* in broiler meat products could be the use of wines and juices as antimicrobial marinade ingredients. It would be a consumer-friendly *Campylobacter* reduction strategy, which could be used in just prior to consumer handling. The antimicrobial property of wine against *C. jejuni* was reported by Carneiro et al. (2008), who suggested that the immersion of food, for example, broiler meat, in wine as a marinade, leads to a reduction in the number of viable *C. jejuni* cells eventually present, thus lowering the risk of cross-contamination of cooked foods. In study III, the red and white wines used had very high bactericidal effects against all the *Campylobacter* strains studied. Other studies have also reported the antimicrobial effects of wines against *Campylobacter*, but with differences in inactivation rates (Carneiro et al. 2008, Birk, Knøchel 2009, Ganan et al. 2009). This might be due to differences in the bacterial strains and growth phases, cultivation media and study conditions, and variations in wine composition. Birk and Knøchel (2009), for example, showed *C. jejuni* to survive 15 min in red wine at 4°C, but when raising the marinating temperature to 42°C, the bacterium was not detectable after 1 min. Grape and tomato juices did not reduce the counts of the *Campylobacter* strains studied as effectively as the wines in study III, even though previous studies have shown that the juices possess antimicrobial activity against different food-pathogens, such as *Listeria* spp. and *E. coli* (Harding, Maidment 1996, Eribo, Ashenafi 2003, Rhodes et al. 2006, Hakovirta 2008). Just and Daeschel (2003), however, also showed that bacteria survive longer in grape juice than in red wine. Since the juices and wines had quite similar pH values in their study and in our study III, it seems that besides the ethanol in wine, also the type of acid and the specific composition of the liquid play a significant role in survival of *Campylobacter*. The commercial marinade in study III had bactericidal effects against all the *Campylobacter* strains studied. This concurred with the results of Perko-Mäkelä et al. (2000), who found that *C. jejuni* were inactivated within 48 h in a plain marinade.

In study III, when testing the antimicrobial effects of wines on the reduction in the *Campylobacter* strains inoculated on the surface of broiler meat, the effects were largely reduced. Correspondingly, Perko-Mäkelä et al. (2000), Björkroth (2005), Birk et al. (2007, 2010) all found the antibacterial effects of marinades or wines on *Campylobacter* less pronounced on broiler meat than the effects in liquid. They all speculated this to be due to the buffering capacity of the meat. In fact, after lowering the pH by adding organic acids onto the meat surface, Birk et al. (2010) observed a rapid rise in pH within a few minutes, which to some extent perhaps neutralized the antibacterial effect. In general, *Campylobacter* strains are recognized as being sensitive to low pH values (Jackson et al. 2009, Silva et al. 2011). The results of study IV also surprisingly indicate that *C. jejuni* NCTC 11168 seems to be slightly more acid-tolerant (at pH 4.0 and pH 5.0) than the close phylogenetic relative *A. butzleri* ATCC 49616. Earlier it has been reported that the responses of *Arcobacter* and *Campylobacter* to different pH values are quite comparable (Chaveerach et al. 2003; Jackson et al. 2009, Hilton et al. 2001, D'Sa, Harrison 2005, Cervenka 2007). However, in the pH studies, as also in the wine studies, the survival of bacteria depends not only on the pH value, but also on the specific composition of the test medium, i.e. the acid used (Cervenka 2007; Shaheen et al. 2007). The temperature used in studies regarding the antimicrobial effects of wines might also affect the results. Birk and Knøchel (2009), for example, submerged pork meat medallions inoculated with *C. jejuni* in red wine at 4ºC or in warm red wine at 42ºC for 15 min prior to storage in red wine at 4ºC and found that under these conditions, the viable counts of *C. jejuni* were reduced by approximately 3.5 log units or close to 6 log after 3 days of storage at 4ºC, respectively. Increasing the temperature and exposure time of *Campylobacter*-inoculated broiler meat to wines over 48 h in study III might have also led to more effective results. Recently, Birk et al. (2010) succeeded in composing a marinade that had both an antimicrobial effect on *C. jejuni* on broiler meat and resulted in an acceptable taste of the prepared meat. This indicates that antimicrobial marinades might still be a potential strategy to control *Campylobacter* in broiler meat products, despite the results gained in study III.

In conclusion, due to the low infective dose of *C. jejuni* in humans (Black et al. 1988) and the modest reductions achieved in the strains of *Campylobacter* species studied, the use of UV irradiation or wines and juices as antimicrobial marinade ingredients cannot be recommended as the primary decontamination methods to control *Campylobacter* in broiler meat. They could, however, be used as part of a sequential risk reduction strategy, because a 2 log reduction in *Campylobacter* levels on broiler carcasses has been predicted to reduce the risk of human exposure and associated illness (Havelaar et al. 2007, Rosenquist et al. 2009, Haughton et al. 2011). Furthermore, Hermans et al. (2011) reported that a 1-, 2- or 3-log reduction in *Campylobacter* counts on carcasses could reduce the incidence by 48%, 85% and 96% respectively.

Specifically, UV is an effective decontamination method for reducing *C. jejuni* on packaging materials and food contact surfaces associated with the preparation of raw broiler meat for sale (Haughton et al. 2011). Wines could perhaps be used as antimicrobial ingredients in broiler meat marinades, together with the addition of further bactericidal agents to control *Campylobacter* in broiler meat. Marinades commonly contain other substances, such as spices, vinegar, etc., that might work synergistically with wines and increase the antimicrobial effects against *Campylobacter* in poultry meat preparations (Carneiro et al. 2008).

6.4 CROSS-PROTECTIVE EFFECT OF STRESS ADAPTATION IN *A. BUTZLERI*

The ability of *Arcobacter* and *Campylobacter* to persist and survive in the broiler production chain when exposed to multiple stress conditions is not fully understood (Humphrey et al. 2007, Collado, Figueras 2011). Adaptation of these bacteria to heat, cold or acid stresses might affect their survival in food processing environments and should be taken to account when designing new food preservation strategies that contain these conditions.

Study IV is the first time cross-protection is reported for *A. butzleri* ATCC 49616. Previously, specific adaptive responses and cross-protection against different stress conditions have been reported for *C. jejuni* (Murphy et al. 2003, Mihaljevic et al. 2007, Reid et al. 2008, Klančnik et al. 2009, Ma et al. 2009). However, other improvements in the bacterial survival after any adaptations were not found in study IV, except the one reported for *A. butzleri* ATCC 49616. Use of bacterial strains always from the same growth conditions and growth phase might explain why specific adaptive responses or more cross-protection were not found in this study. Murphy et al. (2003), for example, showed that the induction of an adaptive tolerance response in *C. jejuni* primarily depended on the growth phase of the cells. In addition, the growth of *C. jejuni* in different media can lead to different adaptive responses (Murphy et al. 2005). Furthermore, study IV was conducted using bacterial collection strains which had been subjected to multiple subcultivations. The survival of wild type strains might be significantly different. Thus, additional work using more bacterial strains from different sources, growth conditions and growth phases is needed. Moreover, the cross-protective effect reported should be studied further at gene expression levels. Interestingly, in study IV the heat stress adapted *A. butzleri* ATCC 49616 cells were more resistant to subsequent lethal acid stress than non-adapted cells only at the 1 h time-point. Ma et al. (2009) also reported that acid, acid and aerobic, or starvation adapted cells survived further acid stress more effectively than the non-adapted cells only in some *C. jejuni* strains used in the study. In addition, the stress-induced adaptive tolerance response in further acid stress was time dependent, i.e. detected only at certain time-points in their study (Ma et al. 2009). Also different periods and conditions of adaptations were used here, compared to earlier studies. Furthermore, in the pH studies, the survival of bacteria depends not only on the pH value, but also on the acid and the test medium used (Cervenka 2007; Shaheen et al. 2007).

In conclusion, heat stress adapted *A. butzleri* ATCC 49616 cells were shown to be more resistant to subsequent lethal acid stress than non-adapted cells at the 1 h time-point. This is the first time cross-protection is reported for *A. butzleri*. This should be taken into account when designing food preservation strategies containing these conditions. Moreover, the cross-protective effect found in *A. butzleri* ATCC 49616 should be investigated further at the gene expression level in order to elucidate the molecular mechanisms behind this phenomenon reported.

7. CONCLUSIONS

- *Campylobacter* prevalence in Finnish retail poultry meat products is low. A high seasonal peak is observed in the products in August. *Campylobacter* was detected in marinated poultry meat products also, which indicates that marinating meat might not affect the survival of *Campylobacter*.

- The PCR assay together with the optimized DNA isolation method used for the detection of *Campylobacter* in marinated and non-marinated retail poultry meat products is faster than microbiological analyses, even though enrichment of the samples is necessary. Thus, it could be used for *Campylobacter*-detection in these types of samples.

- UV irradiation reduced the counts of *C. jejuni* E1 1347 in broiler meat, skin or carcasses only modestly, but it did not affect the sensory quality of broiler meat.

- The exposure of the strains of *Campylobacter* species studied to wines significantly reduced the number of viable cells. However, the antimicrobial activity of wines was strongly reduced when testing their effects on the *Campylobacter* strains inoculated on broiler meat. Juices did not reduce the bacterial counts as effectively as the wines in liquid conditions.

- Due to the low infective dose of *C. jejuni* in humans and the modest reductions achieved for the strains of *Campylobacter* species studied, the use of UV irradiation or wines and juices as antimicrobial marinade ingredients cannot be recommended as the primary decontamination methods to control *Campylobacter* in broiler meat, but might be used as part of a sequential risk reduction strategy to reduce the counts of *Campylobacter*. UV irradiation might be used in combination with other decontamination techniques, together with proper processing plant sanitation and hygiene. Wines could be used as antimicrobial ingredients in broiler meat marinades, together with the addition of further bactericidal agents.

- Heat stress adapted *A. butzleri* ATCC 49616 cells were shown to be more resistant to subsequent lethal acid stress than non-adapted cells at the 1 h time-point. During this study, cross-protection is reported for *A. butzleri* for the first time. This phenomenon should be taken into account when designing food preservation strategies containing these conditions.

- To further evaluate the significance of poultry meat as a source of *Campylobacter* in Finland, their occurrence in the meat products should be quantified. Despite the attempts of this study, no sufficiently effective way to reduce the counts of *Campylobacter* in broiler meat was found. Thus, further decontamination methods should be studied in the future. Moreover, the cross-protective effect found in *A. butzleri* ATCC 49616 should be investigated further at the gene expression level in order to elucidate the molecular mechanisms behind this phenomenon reported.

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PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products

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ABSTRACT

During a period of 9 months, 194 marinated and non-marinated poultry products were collected from retail shops in a defined area in Western Finland and tested for *Campylobacter* spp. using a conventional enrichment culture and Polymerase Chain Reaction (PCR) method. For marinated poultry products, the study involved modification of a commercial DNA isolation method. Using either a conventional culture or PCR method, a total of 25 (12.9%) of all investigated samples were *Campylobacter* positive. In marinated poultry products, *Campylobacter* was detected at a prevalence of 21.1% and 9.5% in turkey and chicken products, respectively. In August, there was a peak with 28.9% positive *Campylobacter* samples. *Campylobacter* inoculation tests were carried out to test the detection limit of both methods. The PCR method used is faster than microbiological analyses. However, enrichment of the samples is necessary due to the low occurrence of *Campylobacter* spp. in retail Finnish poultry products.

1. Introduction

Campylobacter is the most common cause of bacterial enteritis worldwide (Rautelin and Hänninen, 2000; Samuel et al., 2004; Schönberg-Norio et al., 2004, 2006). In Finland, according to the National Infectious Disease Registry, the incidence of campylobacteriosis has increased steadily reaching 4003 and 3439 infections in 2005 and 2006, respectively, with 65 human cases/100,000 inhabitants in 2006 (Anonymous, 2005, 2006a). These high figures make *Campylobacter* nowadays the leading cause of bacterial enteric infections in Finland. Epidemiological studies have underlined handling and consumption of undercooked poultry meat as one of the most important sources of human campylobacteriosis (Evans et al., 2003; Potter et al., 2003; Luber and Bartelt, 2007). Limited studies have been published on the prevalence of *Campylobacter* in poultry meat at the Finnish retail level. Hänninen et al. (2000) studied the prevalence of *Campylobacter* in poultry products in the Helsinki area between June and September from 1996 to 1998 and found 12–21% *Campylobacter* positive samples in each year studied. In summer 2004, the percentages of *Campylobacter* positive fresh broiler and turkey meat at the Finnish retail level were 20.2% and 19.2%, respectively (Anonymous, 2006a–c). In contrast, studies from other countries report a high occurrence of *Campylobacter* at the retail level, for example, 71.3% in the UK (Philipps, 1998), 79.4% in Spain (Mateo et al., 2005) and 64.7% in Japan (Sallam, 2007).

There is a wide variety of poultry products available on the Finnish retail market including fresh and modified-atmosphere-packaged (MAP) products with or without spices. Approximately 80% of these products are sold marinated (Björkroth, 2005). Marinating, in this context, means salting and mixing the meat with water–oil-based, spiced sauces. High NaCl concentration, low pH and the addition of different spices to the marinade prevent the growth of spoilage bacteria, thus increasing the shelf-life of the meat products. However, marinating poultry meat does not decrease pathogenic bacteria such as *Campylobacter* (Evans et al., 1998; Perko-Mäkelä et al., 2000).

Since the popularity and the variety of marinated poultry products in Finland is increasing rapidly every year, reliable methods for the detection of *Campylobacter* in these products are of interest to laboratories in routine work and research. Traditional conventional culture methods include enrichment and plating steps followed by isolation of the bacterium and biochemical identification of the isolate. These methods are laborious, time consuming and costly. Recently designed polymerase chain reaction (PCR) methods have been found to be faster, more specific and sensitive for the detection of *Campylobacter* in naturally contaminated retail samples (Denis et al., 2001; Wong et al., 2004; Mateo et al., 2005; Sallam, 2007). However, several substances in foods can be inhibitory for the PCR. Lilja and

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Hänninen (2001) reported problems in the preparation of marinated chicken samples prior to PCR analysis. Thus, it is important to neutralize such substances by using effective DNA purification protocols or PCR facilitators.

The aim of the present study was to determine the occurrence of *Campylobacter* in naturally contaminated poultry products at the retail level in Western Finland. Special attention was paid to the wide variety of marinated products available on the market. For the detection of *Campylobacter*, a PCR assay has been compared with the conventional culturing method. The original protocol for the DNA isolation using a commercial kit was modified.

2. Materials and methods

2.1. Sampling

A total of 194 raw chilled poultry products (136 chicken samples, 56 turkey samples and two samples including both chicken and turkey meat) were randomly selected between January and September 2006 from different local retail shops in a defined area in Western Finland. All products were packed in Finland, but in 11, nine and two samples, the meat originated from Denmark, Brazil and France, respectively. All samples were transported immediately to the laboratory and kept at 4±3 °C until being analyzed within 24 h of purchase. Between January and June, ten samples were analyzed once a month and from July to September, 15 samples were analyzed three times a month.

2.2. Sample description

The product types and the numbers of samples in each group are presented in Table 2. According to the manufacturers, the meat concentration in all samples varied from 66% to 100% and the salt concentration from 0.6% to 1.6%. The term natural product in this study refers to a non-marinated product of 100% meat without any added substances. Lightly salted products are those with a meat content of 66–80%, a salt concentration of 1% and the addition of water, glucose and food additives. The term "marinated" includes all products with an oil- and/or water-based marinade and a blend of spices with 0.9–1.6% salt. In 47 of all 136 marinated products, honey was the most popular flavor in the marinade. Other common flavors were citrus fruits, peppers, herbs, garlic and barbecue spice. The term "spiced" refers to products with blended spices, salt (0.8–1.4%) and often other ingredients such as flour and breadcrumb rubbed onto the meat surface. The meat content in the marinated and spiced products varies from 66% to 97% and several food additives like stabilizers, antioxidants, acidity balancing agents, preservatives, thickening and flavoring agents are added. These product types also contain maltodextrin, yeast extract and other flavor enhancers. In addition, modified starch, barley or wheat may be added. All the products were packed under a modified atmosphere consisting of carbon dioxide and nitrogen in different proportions and had a shelf life of up to 10 days.

2.3. Culture method for the detection of Campylobacter in poultry samples

Microbiological analyses of the samples were based on a modified method of the National Committee of Food Analyses (2007).

Each sample was aseptically removed from the package and placed in a Stomacher bag (Seward BA6041, Worthing, UK). Equal amounts of a weighed sample and Buffered Peptone Water (BPW) (LAB46, LabM, Lancashire, UK) were mixed with a minimal amount of 300 g of meat in 300 ml of BPW (LabM). The bag was shaken manually for 3 min. For the enrichment, 25 ml of the suspension was re-suspended in 225 ml of Bolton broth (LAB135, LabM) with 5% lysed horse blood and selective supplement (LX132, LabM) and incubated in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2), generated by CampyGen™ (Oxoid CN0035) at 42 °C for 24 h. A loopful of the enrichment culture (10 μl) was streaked on an modified charcoal cefoperazone deoxycholate agar plate (mCCDA) (CM 739, Oxoid, Basingstoke, UK) supplemented with SR 155 (Oxoid) and incubated microaerobically at 42 °C for 48 h. Presumptive *Campylobacter* colonies on mCCDA plates were further identified according to the method of the National Committee of Food Analyses (2007). To test their ability to grow aerobically, they were subcultured onto blood agar plates (CASO agar, Casein-Peptone Soymeal-Peptone, Merck, Darmstadt, Germany with 5% bovine blood) and incubated aerobically at 37 °C for 24 h. Strains were stored at −80 °C in Brucella broth (Scharlau Chemie, Barcelona, Spain) containing 15% glycerol.

2.4. PCR method for the detection of Campylobacter in poultry samples

For the PCR sample, 1.5 ml of the rinsing fluid was centrifuged at 1000 rpm for 8 min at 4 °C. The middle aqueous layer was removed carefully to avoid any fat and placed into an unused Eppendorf tube. After centrifugation at 13,000 rpm for 8 min at 4 °C, the supernatant was removed carefully and the pellet was frozen at −70 °C.

For the PCR of the enriched sample, 1 ml of enrichment culture was collected after 24 h incubation. The subsample was centrifuged at 13,000 rpm for 8 min at room temperature. The supernatant was removed carefully and the pellet was frozen at −70 °C.

2.4.1. DNA isolation

DNA isolation from the frozen pellet was carried out using a DNA isolation kit MagneSil® KF (MD1460, Genomic System, Promega, Madison, WI, USA) with a Dynal MPC®-S magnetic stand (Dynal Biotech, Oslo, Norway). The instructions of the supplier were modified and optimized for DNA isolation by hand using a magnetic stand. A 200 μl lysis buffer and 75 μl magnetic beads were added to an Eppendorf tube containing the pellet. The mixture was vortexed vigorously four times during a 5 min period at room temperature before placing the tube in a magnetic stand with the magnet for 30 s. The magnet was taken out after the liquid was removed from the tube. The particles were washed twice with 185 μl of salt washing buffer and twice with 200 μl of ethanol washing buffer. The tube was then placed in a 72 °C heat block for 5 min with an open lid for ethanol dehydration. The particles were re-suspended in 100 μl of sterile water and replaced in a 72 °C heat block for another 5 min with the lid closed. The tube was mixed with vortex and placed in the magnetic stand for 30 s. The liquid was removed from the tube to be frozen at −20 °C.

2.4.2. PCR assay

The detection of *Campylobacter* in the samples was based on amplification of the 16S rRNA gene (Linton et al., 1996) using two sets of oligonucleotide primers. The first set was C412F 5′-GGA TGA CAC TTT TCG GAG C-3′ (Linton et al., 1996) and 16S rRNA-campR2 5′-GGC TTC ATG CTC TCG AGT T-3′ as described by Lund et al. (2004). The second set was MD16S1, 5′-ATC TAA TGG CTT AAC CAT TAA AC-3′ and MD16S2, 5′-GGA CGG TAA CTA GTT TAG TAT T-3′ as described by Denis et al. (1999). For detection of the internal control the primers Yers F8 5′-CGA GGA GGA AGG GTT

Table 1
List of strains used for validation of specificity of the 412F-16S rRNA-campR2 primer set

Species	Strain	Species	Strain
Campylobacter jejuni	CCUG 11284	*Campylobacter*	CCUG 18267
Campylobacter jejuni	CCUG 24567	*Campylobacter lari*	CCUG 15035
Campylobacter jejuni	CCUG 10940	*Campylobacter lari*	CCUG 12774
Campylobacter jejuni	CCUG 12778	*Campylobacter lari*	CCUG 18294
Campylobacter jejuni	DCC[a] 42	*Campylobacter lari*	DCC 50
Campylobacter jejuni	DCC 43	*Campylobacter lari*	DCC 29
Campylobacter jejuni	DCC 44	*Campylobacter lari*	DCC 33
Campylobacter jejuni	DCC 45	*Campylobacter helveticus*	CCUG 30682
Campylobacter jejuni	DCC 47	*Campylobacter helveticus*	CCUG 30683
Campylobacter jejuni	DCC 48	*Campylobacter helveticus*	CCUG 30563
Campylobacter jejuni	DCC 49	*Campylobacter helveticus*	CCUG 30564
Campylobacter jejuni	DCC 52	*Campylobacter helveticus*	CCUG 30565
Campylobacter jejuni	DCC 22	*Campylobacter helveticus*	CCUG 30566
Campylobacter jejuni	DCC 27	*Campylobacter helveticus*	CCUG 34016
Campylobacter jejuni	DCC 34	*Campylobacter hyointestinalis*	CCUG 14169
Campylobacter jejuni	DCC 40	*Campylobacter hyointestinalis*	CCUG 34538
Campylobacter jejuni	DCC 41	*Campylobacter sputorum*	CCUG 37579
Campylobacter coli	CCUG 11283	*Campylobacter concisus*	CCUG 13144
Campylobacter coli	CCUG 33450	*Campylobacter curvus*	CCUG 13146
Campylobacter coli	DCC 36	*Campylobacter mucosalis*	CCUG 6822
Campylobacter coli	DCC 37	*Campylobacter fetus*	CCUG 6825A
Campylobacter coli	DCC 38	*Arcobacter cryaerophilis*	CCUG 17801
Campylobacter coli	DCC 39	*Arcobacter skirrowii*	CCUG 10374
Campylobacter coli	DCC 46	*Arcobacter butzleri*	CCUG 30485
Campylobacter coli	DCC 51	*Helicobacter pylori*	DCC 35
Campylobacter coli	DCC 28	*Helicobacter pullorum*	DCC 53
Campylobacter coli	DCC 18	*Enteroccocus faecalis*	CCUG 19916
Campylobacter upsaliensis	CCUG 23626	*Escherichia coli*	CCUG 17620
Campylobacter upsaliensis	CCUG 14913	*Streptococcus aureus*	CCUG 17621
Campylobacter upsaliensis	CCUG 24571,	*Staphyloccus bovis*	CCUG 17828
Campylobacter upsaliensis	CCUG 24803	*Salmonellatyphimurium*	DVI-Å[b] 19
Campylobacter upsaliensis	CCUG 23017	*Salmonella enteritidis*	DVI-Å20
Campylobacter upsaliensis	CCUG 20818	*Proteus mirabilis*	CCUG 34293
Campylobacter lari	CCUG 23947	*Bordetella bronchiseptica*	DVI-Å50
Campylobacter lari	CCUG 20575	*Citrobacter freundii*	DVI-Å22

[a] DVI culture collection.
[b] DVI-Å: In house reference strain.

AAG TG-3′ and Yers R10 5′-AAG GCA CCA AGG CAT CTC TG-3′ according to Gibello et al. (1999) slightly modified were used. All the primers were synthesized by Oligomer Oy (Helsinki, Finland).

2.4.3. PCR amplification

The PCR conditions used in the present study are described by Lund et al. (2003) with a few modifications. Briefly, the PCR amplification was performed in 50 μl volumes containing 5 μl of the DNA, 25 μl of a PCR master mix (Promega, Madison, WI, USA), 1 μl of a 25 mM $MgCl_2$ solution, 0.5 μl of a 10 mg ml^{-1} BSA solution (New England Biolabs, Ipswich, MA, USA), 20 pmol of each of the *Campylobacter* primers and 5 pmol of each of the internal control primers.

The PCR was performed in a Peltier Thermal Cycler (PTC-200; MJ Research Inc., Watertown, MA, USA) and the conditions were one cycle of 95 °C for 2 min, 58 °C for 1 min, 72 °C for 1 min, followed by 34 cycles of 95 °C for 15 s, 58 °C for 40 s and 72 °C for 40 s. The last elongation step lasted 5 min. The PCR product was loaded onto a 2% agarose gel (1.35% SeaKem® LE Agarose and 0.65% NuSieve® GTG Agarose, Cambrex Bio Science, Rockland, ME, USA) containing 0.1 μg ml^{-1} ethidium bromide. A DNA molecular weight marker 100 bp low ladder (P1473, Sigma-Aldrich, Saint Louis, MO, USA) was included in each gel. The gel was photographed under UV light (Alpha DigiDoc, Alpha Innotech, San Leandro, CA, USA). The PCR reaction for each sample was performed 1–3 times with each primer set and considered positive if both primer sets gave a distinct band of the right size (857 bp) or at least one primer set gave a positive reaction twice. Samples with no internal control band were run again using a tenfold dilution of DNA.

2.4.4. Control strains

For PCR, *Campylobacter jejuni* EELA 49 strain (isolated from a Finnish broiler carcass) was used as a positive control. As negative controls, sterile water in the PCR method and Bolton broth (LabM) in the culture method were used. An internal control was added to the mastermix. Briefly, DNA from the bacterium *Yersinia ruckeri* (DVI-Å83) was isolated using the MagneSil KF (MD1460, Genomic System, Promega, Madison, WI, USA) as described above. Approximately 25 pg *Y. ruckeri* DNA was added to the mastermix before aliquoting into tubes (Lund et al., 2004; Lund and Madsen, 2006).

The strains used for validation of specificity of the C412F-16S rRNA-campR2 primer set and their sources are listed in Table 1. Strains were stored at −80 °C in brain heart infusion broth (Difco, Detroit, MI, USA) containing 20% glycerol. For testing the specificity of the primers used in the assay, DNA was isolated directly from the storage medium by centrifugation of 0.1 ml of the medium at 15,870 rpm for 7 min and then the pellet was subjected to DNA extraction as described before. Approximately 1ng of DNA was used per PCR.

2.4.5. Comparison of the detection limit between the culture and PCR method

A tenfold dilution series of a *C. jejuni* broth culture was used to determine the detection limit of the culture and the PCR method.

Table 2
Types of Finnish retail poultry products and *Campylobacter* positive samples

Product type	No. of samples positive by culture/ PCR	No. of samples positive[a]/No. of samples tested			
		Slices and barbecue sticks	Breast fillet and fillet steaks	Breasts, legs drumsticks and wings incl. bones and skin	All
Natural		0/3	2/4	2/8	4/15
Lightly salted		0/1	0/0	0/0	0/1
Spiced		0/1	0/6	7/18	7/25
Marinated		3/51	1/24	5/20	9/95
Total chicken samples		3/56	3/34	14/46	20/136
Natural		0/8	0/7	0/0	0/15
Lightly salted		0/0	0/1	0/0	0/1
Spiced		0/0	0/1	0/0	0/1
Marinated		4/19	0/20	0/0	4/39
Total turkey samples		4/27	0/29	0/0	4/56
Marinated		1/2	0/0	0/0	1/2
Total mixed chicken and turkey samples		1/2	0/0	0/0	1/2
Total samples	18/24	8/85	3/63	14/46	25/194

[a] No. of samples tested positive by microbiological method and/or PCR method.

100 μl of each dilution from 10^{-1} to 10^{-7} was plated out for counting the colony forming units (cfu) of the stock solution. Seven samples of 100 g poultry meat slices and 42 g of plain marinade were placed in a Stomacher bag (Seward BA6041). One ml of each dilution of *C. jejuni* broth culture was mixed with 100 ml of BPW (LabM) and this mixture was added to the samples. All samples were subjected to the cultural detection and PCR procedures as described above. This procedure was repeated once.

2.5. Data management and calculations

For data management and calculations, Microsoft® Excel 97 SR 2 and SAS® Systems vers. 8 (Cary, NC, USA) were used. The level of agreement according to precision was expressed as the kappa statistic, defined as the proportion of potential agreement beyond chance exhibited by two tests. Diagnostic specificity was calculated as: $d/(b+d)$ where d is the number of samples negative both by PCR and by culture and b is the number of samples positive by PCR, but negative by culture. The level of agreement between two tests was calculated as: $(a+d)/n$, where a is the number of samples positive both by PCR and by culture, d is the number of samples negative by both methods and n is the total number of samples under examination (Smith, 1995; Martin et al., 1997).

3. Results

3.1. Prevalence of Campylobacter in poultry products

Using either a conventional culture or PCR method, a total of 25 (12.9%) of 194 investigated samples were *Campylobacter* positive (Table 3). The isolation rates from the different product types are listed in Table 2. Out of 136 chicken and 56 turkey products, 20 (14.7%) and four (7.1%) samples respectively were *Campylobacter* positive. One of the two mixed chicken and turkey samples was positive for *Campylobacter*. *Campylobacter* was detected in four (13.3%) of the 30 natural and 14 (10.3%) of the 136 marinated poultry products. Seven (26.9%) of the 26 spiced products were positive, all being chicken with skin and bone. No *Campylobacter* was detected in two lightly salted products. The occurrence of *Campylobacter* in chicken slices and barbecue sticks was 9.4%, in chicken breast fillets 4.7% and chicken products with skin and bone 30.4%. *Campylobacter* was not detected in any of the 22 poultry products with meat of foreign origin.

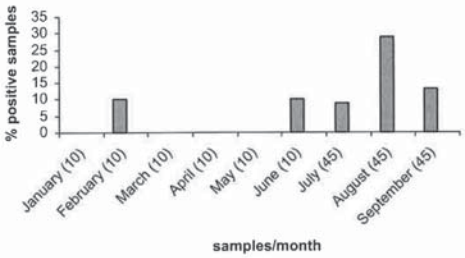


Fig. 1. Monthly distribution of *Campylobacter* positive samples in Finnish retail poultry products from January to September 2006. The numbers in parenthesis represent the number of samples taken per month.

In August, there was a peak with a 28.9% prevalence of *Campylobacter* in 45 investigated samples (Fig. 1). Between January and May, *Campylobacter* was detected in only one of 50 samples.

3.2. Comparison of the culture and PCR method

Eighteen (9.3%) of 194 samples were positive using the conventional culturing method and 24 (12.4%) were positive using the PCR method for *Campylobacter* (Table 3). The results of the culture and PCR were concordant in 186 samples, representing 96.4% of the samples. One sample (1/18), gave a negative result for PCR when the result of the culture method was positive. Seven samples gave a positive result when the culture result was negative (7/176). Approximately 400 bp of the PCR product from five of these samples were sequenced and all sequences were 99% or 100% equal to *C. jejuni*. The diagnostic specificity for the comparison of the PCR to culture by selective enrichment was 0.96 with a level of agreement of 0.96.

Table 3
Comparison of PCR results and culture for the detection of the *Campylobacter* in marinated and non-marinated poultry products after enrichment

PCR	Culture		
	Positive	Negative	Total
Positive	17	7	24
Negative	1	169	170
Total	18	176	194

Table 4
Comparison of detection limit between culture methods and PCR methods

Size of the inoculum in spiked samples (cfu/ml)	Direct culture	Direct PCR	Enrichment culture	Enrichment PCR
7×10^5	+	+	+	+
7×10^4	+	+	+	+
7×10^3	+	+	+	+
7×10^2	+	+	+	+
70	+	–	+	+
7	–	–	+	+
0.7	–	–	+	+

3.3. Specificity of the PCR assays

The specificity of the C412F-16S rRNA-campR2 primer set was tested against a panel of *Campylobacter* and non-*Campylobacter* DNA templates (Table 1). The PCR assay detected *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. helveticus*, and *C. hyointestinalis*, but none of the other *Campylobacter* species tested. No signal was observed for any of the *Arcobater*, *Helicobacter*, or other non-*Campylobacter* species tested. The specificity of the MD16S1–MD16S2 PCR assay was tested by Denis et al. (1999). The assay detected all tested strains of *C. jejuni*, *C. coli* and *C. hyoilei*, but gave no reaction for non-*Campylobacter* strains tested in the study (Denis et al., 1999).

3.4. Comparison of the detection limit between the culture and PCR method

Table 4 shows the results of direct and enrichment culture of spiked samples as well as PCR performed on DNA isolated directly from the same spiked samples or from the enrichments culture of the samples. The detection limit of both enrichment culture and enrichment PCR was less than 1 cfu/ml of sample rinse, while the detection limit of direct culture was 70 cfu/ml. For the direct PCR detection the limit was 700 cfu/ml of sample rinse.

4. Discussion

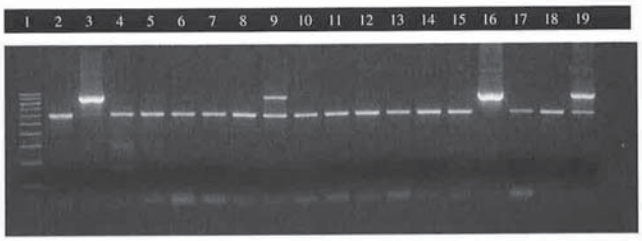
The low prevalence of *Campylobacter* in retail poultry products observed in the present study is consistent with earlier studies carried out in Finland (Hänninen et al., 2000; Anonymous, 2006a). However, these findings are relatively low compared to other countries. In Germany, Luber et al. (2005) reported the occurrence of 67.6% and 11.3% *Campylobacter* on the surface and in the deep muscle tissue of broiler legs respectively. Alter et al. (2004) detected 6.2% and 30.3% *Campylobacter* in 419 turkey and 198 chicken retail products respectively. Nielsen et al. (2006) investigated Danish retail poultry products, including domestic and imported meat, and found *Campylobacter* in 38.7% of 460 chicken and in 27.5% of 204 turkey products. Dominguez et al. (2002) reported 49.5% *Campylobacter* occurrence in 198 chicken meat samples from the Spanish retail market. The low occurrence of *Campylobacter* in Finnish retail poultry products obviously reflects the low prevalence of the organisms in the broiler slaughter batches in Finland. Prevalences between 2.9% and 7.4% have been reported from examination of all broiler slaughter batches during the summer months, when the prevalence is highest in Finland (Perko-Mäkela et al., 2002; Anonymous 2006b, c).

In August, there was a peak with a 28.9% prevalence of *Campylobacter* (Fig. 1). A seasonal variation in chicken flocks has also been observed in the other Nordic countries (Wedderkopp et al., 2000; Bang et al., 2003; Hansson et al., 2004; Hofshagen and Kruse, 2005) and the Netherlands (Bouwknegt et al., 2004). In contrast, Logue et al. (2003) discovered *Campylobacter* more frequently in the cooler months (winter and early spring) in the US. They suggested that the difference in the seasonal occurrence of *Campylobacter* in poultry might be associated with the geographical locations where sampling occurred.

In the present study, *Campylobacter* was also detected in marinated poultry products with a prevalence of 21.1% and 9.5% for turkey and chicken products respectively. Atanassova et al. (2007) reported six out of 16 marinated turkey products from German retail shops as *Campylobacter* positive. Perko-Mäkelä et al. (2000) studied the survival of *C. jejuni* in plain marinade and in both marinated and non-marinated chicken drumsticks and meat slices. In the marinade, a decrease of the inoculated *C. jejuni* level was observed; however, there was no difference between the marinated and non-marinated meat. The authors concluded that marinating may not have an effect on the survival of *Campylobacter*. This may be due to the buffering capability of meat quickly neutralizing the pH of the acidic marinade (Perko-Mäkelä et al., 2000). However, in the present study no *Campylobacter* was detected in 22 marinated sliced chicken products. All these samples were from meat of foreign origin. Foreign meat is frozen when imported to Finland which could be the reason that these samples were negative for *Campylobacter*.

The diagnostic specificity in the comparison between the PCR and the cultural detection by selective enrichment was 0.96 with a level of agreement of 0.96. This is a good agreement between the two methods. One sample of 18 gave a negative result in PCR when the result of the culture method was positive. This false-negative result may be explained by the fact that the size of the subsample used for the culture method is larger than in the PCR method; 25 ml of the rinsing fluid enriched in 225 ml Bolton broth compared to 1 ml for DNA extraction in the PCR method. However, for this reason comparing direct PCR with enrichment culture may be difficult.

Seven samples gave a positive result with PCR after enrichment, whereas the culture result was negative. However, sequencing of the PCR product from five of these samples gave sequences being 99% or 100% equal to *C. jejuni*. The reason for these negative culture results might be the abundant growth of the background flora on mCCDA plates. Six of the seven samples were products with skin likely to contain more contaminating flora than samples without skin (Josefsen et al., 2003). Background flora was detected in 5% of all samples and sometimes it was so abundant that it made the detection of *Campylobacter* impossible. Mateo et al. (2005) reported the same problem with overgrowth in 52.9% of 68 samples and identified *Escherichia coli* in some cases. However, they used Preston broth for selective enrichment. Bolton broth proved to be the best compromise between the inhibition of competitors and growth of *Campylobacter* (Baylis et al., 2000), but this may depend on the material investigated (Josefsen et al., 2003; Tangvatcharin et al., 2005).



Lane 1: 100 bp-DNA molecular weight marker; lane 2: PCR-negative control (internal control); lane 3: PCR-positive control (857 bp); lanes 4 – 8: *Campylobacter*-negative samples; lane 9: *Campylobacter*-positive sample; lanes 10 – 15: *Campylobacter*-negative samples; lane 16: *Campylobacter*-positive sample; lanes 17 – 18: *Campylobacter*-negative samples; lane 19: *Campylobacter*-positive sample.

Fig. 2. Gel electrophoresis patterns of PCR products of *Campylobacter* from enrichment samples of naturally contaminated marinated and non-marinated poultry products at the Finnish retail level using C412F and 16S rRNA campR2 primers.

Susceptibility to inhibitory substances, which can be found in high levels in foods, is a great disadvantage of PCR (Abu Al-Soud and Radstrom, 1998; Abu Al-Soud and Radstrom, 2000; Rossen et al., 1992). Lilja and Hänninen (2001), studying marinated chicken products and using the Buoyant Density Centrifugation (BDC) method for sample preparation, reported problems probably caused by emulsifiers used in the oil-spice. Rossen et al. (1992) used a pre-treatment step with hot sodium hydroxide/sodium dodecyl sulfate to reduce the effect of food inhibitory substances. In the present study, a pre-centrifugation step was performed in order to exclude most of the lipids and fat from the marinade and the chicken skin. As DNA isolation was performed manually with a DNA isolation kit for automated DNA isolation, further optimization compared to manufacturer's instructions was necessary to make the manual DNA isolation as sensitive as the automated isolation. Vigorous vortexing of the samples in lysis buffer was found to be the most important step. To optimize DNA isolation from marinated poultry products, one possibility could be to add fat digesting enzymes to the bacterial pellet just prior to DNA isolation. A nested PCR method could lower the detection limit, however, when the number of *Campylobacter* is very low, it is a question of statistics if any bacteria will appear in a 1 ml sample.

To control the PCR reaction in the different samples studied in this assay, an internal control PCR was run simultaneously with the target DNA (Fig. 2). In both PCR reactions, performed on DNA isolated directly from the samples and on DNA isolated from the enrichment media, the internal control gave a band of same intensity showing no evidence of inhibition of the PCR reaction. However, the detection limit of the direct PCR was about 700 cfu/ml (Table 4). This is high compared to other direct PCR assays for *Campylobacter*. Lund et al. (2003) reported a detection limit of approximately 40 cfu/ml in fecal material and Yang et al. (2003) of 100 cfu/ml in the same material using a real-time PCR assay. As inhibition of the PCR reaction does not seem to be the problem, it may also be possible that *Campylobacter* are preferably located in the fatty part of the sample and as this part is removed before DNA isolation many bacteria might be lost. On the other hand, the fat and or protein still present in a sample after pre-treatment could interfere with DNA isolation. As the detection limit of the present direct PCR was too high compared to the normally quite low amount of *Campylobacter* in food and retail poultry samples, it was necessary to perform a combination of enrichment and PCR assay.

In the present study, we used a new combination of primers (C412F and 16S rRNA campR2). A tendency was seen that this primer set captured more of the samples that were culture negative and negative with the MD16S1 and MD16S2 primers (results not shown). However, the differences were not statistically significant.

5. Conclusions

The PCR method used shortens time compared to microbiological analyses and can be therefore used for detection of *Campylobacter* spp. in poultry products. However, enrichment of the samples is necessary due to the low occurrence of *Campylobacter* spp. in Finnish poultry products at the retail level.

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II

ARTICLE II

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"Use of ultraviolet irradiation to reduce *Campylobacter jejuni* on broiler meat"

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PROCESSING, PRODUCTS, AND FOOD SAFETY

Use of ultraviolet irradiation to reduce *Campylobacter jejuni* on broiler meat

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ABSTRACT The effects of UV irradiation at a wavelength of 254 nm on the survival of *Campylobacter jejuni* on the surfaces of broiler meat, skin, and carcasses were studied. On broiler carcasses, the effects of UV were also studied in combination with activated oxygen. The surfaces were inoculated with varying counts of *C. jejuni* and treated with UV irradiation using doses ranging between 9.4 and 32.9 mW/s per square centimeter. The log reductions in *C. jejuni* counts were determined by dilution plating. The effects of both treatments on the sensory quality of broiler meat, including visual appearance, odor, and fatty acid composition, were also evaluated. On broiler meat, the maximum reduction achieved was 0.7 log and on broiler skin 0.8 log. On broiler carcasses, the maximum reduction using UV irradiation was 0.4 log, and using UV in combination with activated oxygen 0.4 log. No significant differences were found in the sensory quality between the samples and the controls. The use of UV irradiation alone or in combination with activated oxygen cannot be recommended as a primary decontamination method for *C. jejuni* on broiler carcasses. The use of these methods in combination with other decontamination techniques, and processing with proper processing plant sanitation and hygiene, might be more effective in reducing the *C. jejuni* counts on broiler carcass surfaces than the use of these methods only.

Key words: *Campylobacter jejuni*, ultraviolet irradiation, broiler meat, decontamination

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INTRODUCTION

During recent years, *Campylobacter* species have been the greatest reported cause of human bacterial gastroenteritis worldwide (Rautelin and Hänninen, 2000; Samuel et al., 2004; Anonymous, 2008a). According to Finland's National Infectious Disease Registry, the incidence of campylobacteriosis reached 4,107 infections in 2007, which is 668 (19%) more infections than reported in 2006 (Anonymous, 2007a). One reason for this was a large outbreak in the Finnish town of Nokia in November 2007, associated with the escape of refined wastewater into the water system (Anonymous, 2008b).

Many epidemiological studies have shown that one of the most important sources of human campylobacteriosis is the consumption and handling of undercooked poultry meat (Rosenquist et al., 2006; Wingstrand et al., 2006). During the slaughter process, poultry carcasses become frequently contaminated with *Campylobacter* spp., especially via leakage of the intestinal contents during defeathering and evisceration (Berndtson et al., 1992; Rosenquist et al., 2006). The nature of poultry processing makes it difficult to prevent cross-contamination between birds and flocks in the slaughterhouse (Borck and Pedersen, 2005). Therefore, one effective approach to reduce contamination could be to decontaminate the poultry carcasses after processing.

Several methods for the reduction of *Campylobacter* spp. on fresh broiler carcasses have been studied, for example using steam or hot water in combination with rapid cooling, chilling or freezing of carcass surfaces (James et al., 2007), spray washing of carcasses with acidified electrolyzed water or sodium hypochlorite solutions (Northcutt et al., 2007), and chilling of broiler carcasses in cold air or ice water (Berrang et al., 2008). Another possibility to reduce contamination is UV irradiation, which is commonly used for the decontamination of packing surfaces or in food-processing environments (Corry et al., 1995; Bolder, 1997; Dinçer and Baysal, 2004). The range of UV radiation that is considered to be germicidal against bacteria is between 220 and 300 nm (UVC), and generally a wavelength of 254 nm is used for decontamination (Guerrero-Beltrán and Barbosa-Cánovas, 2004). This range of UV contains high-energy photons that generate pyrimidine dimers and denature bacterial DNA, leading to the destruction of bacteria by degradation of the cell walls (Guerrero-Beltrán and Barbosa-Cánovas, 2004). The benefits of UV irradiation are that it is a nonthermal, chemical-free process that leaves no residues and the equipment can be easily installed at relatively low cost (Wallner-

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Pendleton et al., 1994; Wong et al., 1998; Lyon et al., 2007).

Several studies have been undertaken to investigate the effect of UV irradiation on food items. It reduced *Salmonella* spp. and *Escherichia coli* O157:H7 on fruits and vegetables (Yaun et al., 2004) and other microorganisms in some liquid foods (Wright et al., 2000; Guerrero-Beltrán and Barbosa-Cánovas, 2004). Stermer et al. (1987) found that UV lowered the counts of bacteria commonly found on beef meat, mostly *Pseudomonas*, *Micrococcus*, and *Staphylococcus* spp., whereas on poultry skin and carcasses, *Salmonella* Typhimurium was diminished (Wallner-Pendleton et al., 1994; Sumner et al., 1996). In a study by Wong et al. (1998), UV reduced *Salmonella* Senftenberg and *E. coli* on pork muscle and skin. Kim et al. (2002) discovered reductions of *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella* Typhimurium on chicken meat after UV irradiation, as did Lyon et al. (2007) on broiler breast fillets for *L. monocytogenes*. Butler et al. (1987) reported inactivation of *Campylobacter jejuni* in liquid samples after UV irradiation. However, to the authors' knowledge, there is no information available on the effects of UV irradiation on *C. jejuni* on broiler meat.

Activated oxygen could be another possible agent to decontaminate broiler meat. Activated oxygen can be created from ordinary oxygen through the addition of energy (natural or generated), which changes the momentum of the electrons in the oxygen, or the oxygen becomes ionized (Seo et al., 2001; Arnold and Mitchell, 2002). Activated oxygen has oxidizing effects on the ions in lipids and on amino acids in bacteria, which leads to degradation of the cell walls. When the ions react with water vapor, the hydroxyl created destroys the DNA of the bacteria (Seo et al., 2001; Arnold and Mitchell, 2002). The use of UV irradiation in combination with activated oxygen could potentially be an effective method for reducing bacteria on broiler meat. However, the effects of activated oxygen alone or in combination with UV irradiation on *C. jejuni* on broiler meat have not been previously studied.

The objectives of this study were to determine a) preliminarily the effects of UV irradiation on *C. jejuni* inoculated on agar plates and b) the effects of UV irradiation on *C. jejuni* inoculated onto the surfaces of broiler meat, skin, and carcasses. In addition, the effects of UV irradiation in combination with activated oxygen on *C. jejuni* inoculated on broiler carcasses, and the effects of the treatments on the sensory quality of broiler meat, have been determined.

MATERIALS AND METHODS

Bacterial Strain

A *C. jejuni* strain E1 1347 was used in all experiments. The strain has been isolated from broiler cecum content at a broiler slaughterhouse. It has been identified according to a modified method of the National Committee of Food Analysis (2007). The strain was maintained at −75°C in *Brucella* broth (02-042, Scharlau Chemie, Barcelona, Spain) containing 15% glycerol.

Preparation of Inoculum

To prepare the bacterial suspension to be used for inoculation of agar plates and of broiler meat and skin, the cells from the frozen stock were plated onto *Campylobacter* blood-free selective medium (modified CCDA-Preston, **mCCDA**; CM0739, Oxoid, Hampshire, UK) containing CCDA selective supplement (SR0155, Oxoid) and incubated at 42 ± 1°C for 22 ± 2 h under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) obtained by CampyGen Compact (CN0020C, Oxoid). After incubation, the resulting growth was suspended into 10 mL of physiological saline (0.85% NaCl). To create the 100-mL bacterial suspension to be used for inoculation of broiler carcasses, the cells from the frozen stock were grown on mCCDA (Oxoid) as described above. The resulting growth was first suspended into 10 mL of buffered peptone water (**BPW**; LAB46, LabM, Lancashire, UK), and then 1 mL of this suspension was resuspended into 99 mL of sterile BPW (LabM).

Inoculation

Agar Plates. Trypticase soy agar II with 5% horse blood (212099, BD, Franklin Lakes, NJ) was used in this experiment. One milliliter of the inoculum prepared in physiological saline was serially diluted in 9 mL of physiological saline (dilutions from 10^{-1} to 10^{-6}), and 12 spread plates from each dilution were prepared.

Broiler Meat and Skin. Fresh broiler meat with or without skin packed in a modified atmosphere was purchased from a local supermarket. One package of meat was used per experiment and was determined to be *Campylobacter*-free by the enrichment PCR method as described in Katzav et al. (2008). Skin samples were cut from the thigh pieces and the meat samples from boneless, skinless breast fillets. The samples were aseptically excised with sterile knives and cut into 2 × 2 cm pieces (4 cm^2) to a thickness of about 0.5 cm. The samples (10 per experiment) were placed flattened out on the bottom of sterile small Petri dishes. The skin samples were placed with the follicle surface up and the meat with the outer surface up. The sample surfaces were flamed with a Bunsen burner for about 5 s and cooled at room temperature for 5 min. Then the samples were inoculated by spreading a 100-μL aliquot of the inoculum prepared in physiological saline evenly over the entire surface of each sample. The samples were allowed to dry for 15 min before exposure to UV.

Broiler Carcasses. Refrigerated fresh broiler carcasses were obtained from a local poultry slaughterhouse. Eight carcasses were used per experiment and were determined to be *Campylobacter*-free by enrichment PCR (Katzav et al., 2008). In one experiment, the carcasses were inoculated individually with the 100-mL

BPW (LabM) bacterial suspensions. Each carcass was massaged manually with the suspension for 3 min in a big Stomacher 3500 bag (BA6042, LabM). After inoculation, the carcasses were aseptically removed from the bags and allowed to dry for 15 min in a laminar flow hood.

Enrichment PCR

In the studies on broiler meat and skin, 20 g of meat was aseptically placed in a Stomacher 400 bag (BA6041, Seward, Worthing, UK) containing 180 mL of selective Bolton broth (LAB135, LabM) with 5% lysed horse blood and selective supplement (LX132, LabM). The samples were shaken manually for 3 min and incubated at 42 ± 1°C for 22 ± 2 h under microaerobic conditions generated by CampyGen (CN0035, Oxoid). In the studies on broiler carcasses, neck skins (each weighing 5 g) from 4 carcasses were pooled to create 1 subsample in 180 mL of selective Bolton broth (LabM) and handled as above. For a PCR sample, 1 mL of Bolton broth (LabM) was collected after enrichment and centrifuged at 11,300 × *g* for 8 min. The supernatant was removed carefully and the pellet was frozen at −75°C. The DNA isolation method and enrichment PCR assays were conducted as described in Katzav et al. (2008).

UV Treatment and Determination of Bacterial Counts

Agar Plates. Three of the 12 spread plates (Trypticase soy agar II with 5% horse blood, BD) prepared from each dilution were not UV-treated (controls) and the other 9 were subjected without a lid to the 3 different doses of UV light (3 plates/dose).

Broiler Meat and Skin. In each experiment, 5 broiler meat and skin samples were not UV-treated (controls) and 5 were UV-treated. One dose was studied per experiment. The tests were replicated 5 times for each 3 UV dose studied. For determination of the bacterial counts, each surface was swabbed with 5 sterile premoistened cotton swabs. The swabs were placed into 5 mL of physiological saline, allowed to soak for 5 min, and removed. One milliliter of the suspension from each sample was serially diluted in 9 mL of physiological saline (dilutions from 10^{-1} to 10^{-6}). Three spread plates (Trypticase soy agar II with 5% horse blood, BD) were prepared from each dilution.

Broiler Carcasses. In 1 experiment, 2 of the carcasses were not UV-treated (controls) and the other 6 were subjected to the 3 doses of UV light studied alone or in combination with activated oxygen (2 carcasses/dose). The tests were replicated 3 times for each of the 3 doses studied. For determination of the bacterial counts, the carcasses were separately manually massaged for 3 min in a Stomacher bag (LabM) with sterile 100 mL of BPW (LabM). One milliliter of the sample was serially diluted in 9 mL of BPW (dilutions from 10^{-1} to 10^{-6}). Three spread plates (mCCDA, Oxoid) from each dilution were prepared.

Culture Detection

All plates were incubated at 42 ± 1°C for 44 ± 4 h under microaerobic conditions obtained by GasPak EZ Campy Container System Sachets (260680, BD). The resulting colony-forming units per milliliter were averaged from the triplicate plates. The colonies were identified by colony morphology and Gram staining.

Determination of Reductions

The log reductions in the experiments were calculated by using the following formula: log reduction = $\log_{10}$ initial concentration − $\log_{10}$ final concentration. The reductions in single experiments on broiler meat or skin per dose were calculated based on the average colony-forming units per milliliter before (n = 5) and after (n = 5) the UV treatment, and the final reductions were based on the average reductions from the test replications (n = 5). On broiler carcasses, the reductions in single experiments were calculated based on the average colony-forming units per milliliter before (n = 2) and after (n = 2 per dose) the UV treatments alone or in combination with activated oxygen, and the final reductions were based on the averages from the results of the test replications (n = 3).

UV Equipment

In the experiments on agar plates and on broiler meat and skin, a UV irradiator (BIOCID 72 IP67, Oy BIOCID Ltd.) with 4 lamps generating 254 nm of wavelength, 5.5 W of UV effect per lamp, and a fre-

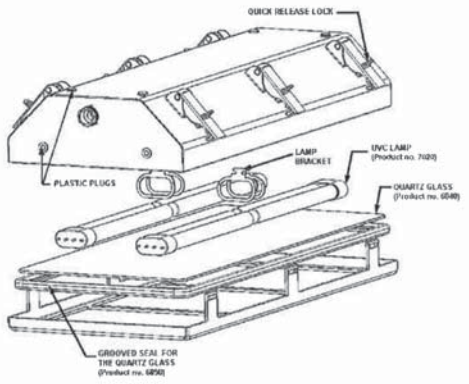

Figure 1. BIOCID 72 IP67 UV (Oy BIOCID Ltd., Vantaa, Finland) irradiator used in the experiments on agar plates and on broiler meat and skin.

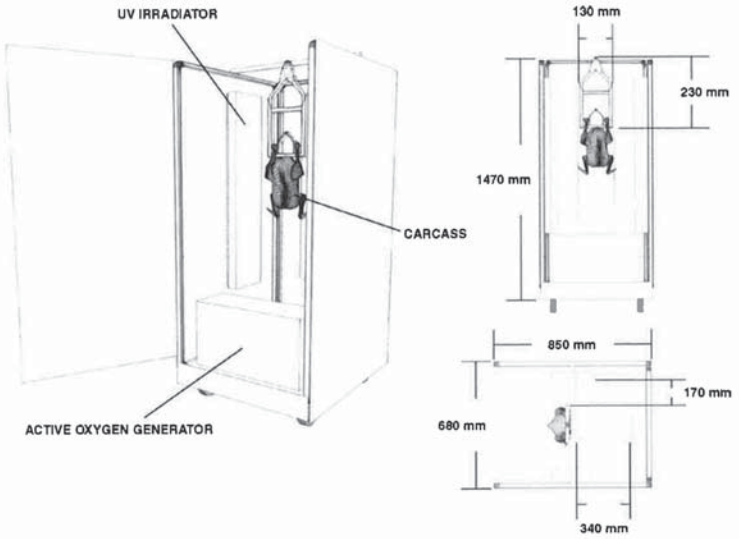

Figure 2. Ultraviolet chamber with 3 UV irradiator units (BIOCID 110 IP55, Oy BIOCID Ltd., Vantaa, Finland) and with an active oxygen generator (BIOCID MX1800-E9-IC) used in the studies on broiler carcasses.

quency of 50 Hz was used (Figure 1). It was placed in a laminar flow hood on metallic stands (height 21 cm) and allowed to warm up for 30 min before the tests. The UV intensity was measured in milliwatts per square centimeter with an UVX radiometer (UVP Inc., Upland, CA), which was calibrated before studies by the UV irradiator manufacturer. The inoculated surfaces received a UV intensity of 2.35 mW/s per square centimeter.

For the studies on broiler carcasses, a UV chamber (Figure 2) was constructed by Oy BIOCID Ltd. Three UV irradiator units (BIOCID 110 IP55, Oy BIOCID Ltd.) were mounted inside the chamber walls (made of stainless steel). Each of the units contained 4 lamps generating 254 nm of wavelength, 16.5 W of UV effect per lamp, and a frequency of 50 Hz. A standard plastic broiler meat hook was placed in the center of the chamber, so that the carcasses received UV light from 3 sides (front, left, and right) in the experiments. After 15 min of warming up, UV intensity was measured in milliwatts per square centimeter with a UVX radiometer (UVP Inc.). The carcasses received a UV intensity of about 1.80 mW/s per square centimeter. The UV chamber contained also an active oxygen generator (BIOCID MX1800-E9-IC, Oy BIOCID Ltd.) on the floor of the chamber. The generator was turned on or off by disconnecting the plug from the socket inside the chamber.

Table 1. The log[1] reductions (mean ± SD) of *Campylobacter jejuni* counts on the surfaces of broiler meat and skin after UV irradiation (n = 5)

UV dose (mW/s per square centimeter)	Broiler meat	Broiler skin
9.4	0.6 ± 0.2	0.8 ± 0.3
18.8	0.7 ± 0.2	0.6 ± 0.2
32.9	0.7 ± 0.2	0.8 ± 0.1

[1]Log_{10} initial concentration − $\log_{10}$ final concentration.

UV Doses

The 3 UV doses studied were determined by multiplying the exposure time (s) by the applied intensity (mW/s per square centimeter; kept constant). They were expressed as milliwatts per second per square centimeter. All doses were selected based on the UV equipment manufacturers' suggestion. Exposure times in the studies on agar plates, broiler meat, and skin were 4 s (dose 9.4 mW/s per square centimeter), 8 s (dose 18.8 mW/s per square centimeter), and 14 s (dose 32.9 mW/s per square centimeter), and in the studies on broiler carcasses, 6 s (dose 10.8 mW/s per square centimeter), 10 s (dose 18.0 mW/s per square centimeter), and 18 s (dose 32.4 mW/s per square centimeter). They were measured with a laboratory timer. Each sample was put at the location with the expected intensity and removed immediately after treatment. Ultraviolet intensity at the expected location of the samples was measured for accuracy and repeatability before and after each trial.

Table 2. The log[1] reductions (mean ± SD) of *Campylobacter jejuni* counts on the surface of broiler carcasses after treatment with UV irradiation alone or with UV in combination with activated oxygen (n = 3)

UV dose (mW/s per square centimeter)	UV	UV + activated oxygen
10.8	0.3 ± 0.1	0.3 ± 0.1
18.0	0.3 ± 0.2	0.3 ± 0.1
32.4	0.4 ± 0.1	0.4 ± 0.1

[1]$\log_{10}$ initial concentration − $\log_{10}$ final concentration.

Sensory Analyses

Refrigerated fresh broiler meat fillets with or without skin were purchased from a local poultry slaughterhouse. The samples were treated with UV alone or with UV in combination with activated oxygen in the UV chamber as described above. Treatment times of 10 and 100 s were used (controls not treated). Each test series contained 12 broiler meat samples either with or without skin. After the treatments, the samples were packed in batches of 2 in vacuum bags and were vacuum degassed and stored at 4 ± 1°C. In the sensory analyses, the changes in color and sensory quality, including visual appearance and odor of the samples, were evaluated on d 0, 2, 5, 7, 9, and 12 after the treatments. The fatty acid composition of the samples was measured on d 0, 5, 7, and 12 after the treatments.

The color of the samples was analyzed using a Minolta CR-200 colorimeter (Minolta Co. Ltd., Ramsey, NJ), which was calibrated before the tests. The sample bags were opened and allowed to warm up to room temperature for 15 min. Then the L (relative lightness of the color), +a (redness), and +b (yellowness) values of the samples were measured. From the samples containing skin, the color readings were measured both from the thick and thin parts of the fillets, and from the skinless samples twice from the top of the fillets. The measurements were done in parallels of 2 and averaged.

The fatty acid composition of the samples was determined using method LA0202 (Anonymous, 2007b) with a Hewlett Packard 5890 gas chromatograph (GMI Inc., Ramsey, MN). The fatty acids were identified by comparing the retention times with the retention times of the standard diagram. The results were reported as area percentages of saturated fat, monounsaturated fat, polyunsaturated fat, and unidentified fat by using a ready formula in Excel (Microsoft Corp., Redmond, WA). The ratio between polyunsaturated and saturated fat was also reported.

Statistical Analyses

The statistical differences between the effects of the 3 doses of UV irradiation on broiler meat and skin were determined by ANOVA, in which the initial concentration was used as a covariate. The statistical differences between the effects of the 3 doses of UV alone or of UV in combination with activated oxygen on broiler carcasses were determined by ANOVA. All analyses were performed by means of SPSS 14.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

In the preliminary test conducted on agar plates, at least a 6.3 log cycle reduction in *C. jejuni* counts was observed with all the UV irradiation doses studied (data not shown). The greatest concentration of *C. jejuni* inoculated on agar plates was $\log_{10}$ 7.0 cfu/mL, and thus UV irradiation was very effective in reducing *C. jejuni*. Butler et al. (1987) reported a similar effect with a 3 log cycle reduction of *C. jejuni* in liquid samples after treatment with UV dose 1.8 mW/s per square centimeter. Other authors also found UV to be effective in reducing bacteria, like *E. coli*, *Salmonella* Senftenberg, *Salmonella* Typhimurium, *Pseudomonas*, *Micrococcus*, and *Staphylococcus* spp., on agar surfaces (Stermer et al., 1987; Sumner et al., 1996; Wong et al., 1998; Kim et al., 2002; Yaun et al., 2003).

On broiler meat, the maximum reduction of *C. jejuni* achieved with UV irradiation was 0.7 log cycles

Table 3. Lightness (L), redness (+a), and yellowness (+b) color values (mean ± SD) of untreated and UV-treated in combination with activated oxygen (AO)-treated broiler fillets without skin after 100-s treatment times (n = 4)

		Color values		
Treatment	Day	L	+a	+b
Control	0	55.25 ± 4.99	4.88 ± 2.63	1.12 ± 2.08
	2	56.94 ± 1.80	3.86 ± 0.90	1.40 ± 0.84
	5	55.56 ± 2.76	3.30 ± 1.08	0.48 ± 0.74
	7	55.12 ± 1.11	5.11 ± 3.33	1.01 ± 1.60
	9	56.74 ± 1.72	3.48 ± 0.70	0.62 ± 1.73
	12	56.45 ± 5.63	4.49 ± 2.79	1.66 ± 1.43
UV 100 s + AO	0	55.08 ± 1.71	2.84 ± 0.35	1.35 ± 0.54
	2	50.96 ± 1.89	5.32 ± 1.37	−0.25 ± 1.17
	5	52.69 ± 0.34	2.13 ± 0.73	0.28 ± 0.17
	7	57.92 ± 0.99	2.05 ± 1.14	1.96 ± 0.70
	9	53.30 ± 2.84	3.81 ± 0.73	1.13 ± 0.91
	12	53.29 ± 0.90	1.74 ± 0.64	−0.33 ± 1.02

Table 4. Lightness (L), redness (+a), and yellowness (+b) color values (mean ± SD) of untreated and UV-treated in combination with activated oxygen (AO)-treated broiler fillets with skin after 100-s treatment times (n = 4)

		Color values		
Treatment	Day	L	+a	+b
Control	0	73.72 ± 5.07	4.24 ± 1.99	4.95 ± 4.22
	2	71.95 ± 3.73	5.25 ± 2.91	8.07 ± 4.12
	5	72.62 ± 2.24	4.31 ± 1.09	5.22 ± 2.03
	7	71.94 ± 3.69	6.72 ± 3.99	8.68 ± 2.58
	9	71.88 ± 3.22	4.40 ± 2.47	5.50 ± 3.02
	12	70.19 ± 5.54	2.92 ± 1.79	3.40 ± 4.81
UV 100 s + AO	0	71.90 ± 3.91	5.91 ± 4.62	6.97 ± 3.73
	2	72.54 ± 2.41	4.98 ± 2.32	6.24 ± 2.99
	5	71.74 ± 4.73	4.19 ± 3.14	5.54 ± 4.73
	7	70.17 ± 2.99	4.42 ± 2.32	5.16 ± 3.80
	9	71.77 ± 4.18	2.84 ± 1.97	4.87 ± 3.93
	12	72.86 ± 3.98	4.02 ± 1.72	7.98 ± 3.82

(Table 1), with a dose of 32.9 mW/s per square centimeter being the most effective. There were statistically significant differences between the effects of the doses 9.4 and 32.9 mW/s per square centimeter ($P = 0.030$). The effects of the 3 doses significantly depended on the concentration of *C. jejuni* initially inoculated onto the meat ($P = 0.029$), which was variable throughout the test replications (between $\log_{10}$ 6.4 and 7.5 cfu/mL). The lower the initial concentration, the better the reducing capacity of UV irradiation was. On broiler skin, the maximum reduction of *C. jejuni* achieved with UV irradiation was 0.8 log cycles (Table 1), with a dose of 32.9 mW/s per square centimeter being most the effective. There were no significant differences between the effects of the UV doses ($P \geq 0.05$). In contrast to meat, the effects of UV irradiation did not significantly depend on the initial concentration of *C. jejuni* ($P \geq 0.05$), although this varied (between $\log_{10}$ 6.6 and 7.5 cfu/mL).

Ultraviolet irradiation was less effective in eliminating *C. jejuni* on broiler meat and skin than on agar plates but appeared to be a little more effective in reducing *C. jejuni* on broiler skin than on meat. One reason could be the dry meat surface due to flaming before inoculation. Ultraviolet irradiation has low penetration capabilities, and maybe in this study the cut edges in the meat created shadows and interfered with the penetration of UV radiation as observed by Lyon et al. (2007). The meat fibers might have separated when the surfaces were swabbed and allowed the swabs to absorb some of the moisture from below the meat surface. Skin did not seem to change to the same extent after flaming, and the bacteria might not have been able to enter the skin as much as the meat. Wong et al. (1998) also reported a more effective reduction of bacteria on pork skin than on meat. The reductions achieved with UV irradiation in earlier studies on meat were more effective compared with this study. Stermer et al. (1987) found that UV reduced the bacteria commonly present on the surface of beef meat about 2 log cycles. Wong et al. (1998) reported that with UV, maximum logarithmic reductions for *Salmonella* Senftenberg on pork muscle were about 2.0 and on pork skin about 4.6, whereas for *E. coli* they were about 1.9 and about 3.3, respectively. Kim et al. (2002) discovered up to 1.3 log cycle reductions of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* Typhimurium on chicken meat after UV treatment, and Lyon et al. (2007) showed about 2 log reductions of *L. monocytogenes* on broiler breast fillets. The lower doses used in the present study and the testing of other bacteria than *C. jejuni* might explain the differences. However, the present results on broiler skin are similar to a study by Sumner et al. (1996), where an 80.5% reduction of *Salmonella* Typhimurium

Table 5. Unknown (U), saturated (S), monounsaturated (M), and polyunsaturated (P) fatty acid compositions (area percentages) and the ratio between polyunsaturated and saturated fat (P:S) of untreated and UV-treated in combination with activated oxygen (AO)-treated broiler fillets without skin after 100-s treatment times (n = 1)

		Fatty acid composition (%)				
Treatment	Day	U	S	M	P	P:S
Control	0	4.2	28.7	46.2	20.9	0.73
	5	6.2	29.6	43.7	20.6	0.69
	8	5.1	29.1	42.5	23.2	0.80
	12	5.7	28.8	44.9	20.6	0.72
UV 100 s + AO	0	4.0	28.1	43.5	24.3	0.87
	5	4.3	28.4	44.8	22.5	0.79
	8	4.6	28.7	41.9	24.8	0.87
	12	5.5	29.5	40.2	24.8	0.80

Table 6. Unknown (U), saturated (S), monounsaturated (M), and polyunsaturated (P) fatty acid compositions (area percentages) and the ratio between polyunsaturated and saturated fat (P:S) of untreated and UV-treated in combination with activated oxygen (AO)-treated broiler fillets with skin after 100-s treatment times (n = 1)

		Fatty acid composition (%)				
Treatment	Day	U	S	M	P	P:S
Control	0	2.9	27.5	49.0	20.6	0.75
	5	2.7	27.3	50.1	19.9	0.73
	8	2.9	28.4	48.1	20.7	0.73
	12	2.4	28.6	48.3	20.7	0.73
UV 100 s + AO	0	3.0	28.3	47.8	20.9	0.74
	5	3.1	27.2	47.4	22.3	0.82
	8	3.6	26.4	48.3	21.8	0.83
	12	2.8	28.8	48.8	19.5	0.68

on poultry skin was found after UV treatment with a dose of 2,000 $\mu W/cm^2$. In our study, the maximum percentage of reduction on broiler skin was 85.3%.

On broiler carcasses, the maximum reduction of *C. jejuni* achieved with UV irradiation was 0.4 log cycles (Table 2). A 61% reduction in *Salmonella* Typhimurium counts on poultry carcasses was observed by Wallner-Pendleton et al. (1994), which is almost similar to our study in which the maximum percentage of reduction on broiler carcasses was 62.7%. It might be that the uneven shape of a carcass creates shadows on the surface, where UV irradiation with its low penetration capabilities does not reach (Lyon et al., 2007). The effects of UV irradiation on *C. jejuni* strains of different origin and at different growth stages might differ greatly (Yaun et al., 2003). The initial concentration of the present inoculants was greater than expected to occur on naturally contaminated carcasses at the slaughterhouse. Aho and Hirn (1988) studied the prevalence of *Campylobacter* spp. in the Finnish broiler chain and found mean $\log_{10}$ 4.5 cfu *Campylobacter* spp. on the surfaces of fresh broiler carcasses. However, their study was conducted only on 3 carcasses. Thus, more information about *Campylobacter* spp. numbers on fresh broiler carcasses in Finland is needed.

On broiler carcasses, the maximum reduction of *C. jejuni* achieved with UV in combination with activated oxygen was 0.4 log cycles (Table 2). A dose of 32.4 mW/s per square centimeter was the most effective in both treatments. There were no statistical differences between the effects of the different treatment doses studied in either treatment type ($P \geq 0.05$). With both treatments, the effects of the doses did not significantly depend on the initial concentration of *C. jejuni* ($P \geq 0.05$), which varied throughout the test replications with UV between $\log_{10}$ 4.5 and 5.3 cfu/mL and with UV in combination with activated oxygen between $\log_{10}$ 5.7 and 6.3 cfu/mL. Using UV alone or in combination with activated oxygen was not as effective in reducing *C. jejuni* on broiler carcasses as UV was on agar plates and on broiler meat and skin. Activated oxygen did not significantly increase the reducing effects of UV radiation. One reason for this could be the short treatment times used in this study.

No significant differences were found in the color (Tables 3 and 4) and sensory quality (data not shown) of the controls and the samples treated with UV or with UV in combination with activated oxygen. There were also no significant differences in the fatty acid compositions of the controls and the samples (Tables 5 and 6). The advantage of UV irradiation having no deleterious effects on the sensory quality of broiler meat has been previously reported by Stermer et al. (1987), Wallner-Pendleton et al. (1994), and Lyon et al. (2007), too.

In conclusion, UV irradiation alone or in combination with activated oxygen had an effect on the survival of *C. jejuni*. Both methods do not affect the sensory quality of fresh poultry. However, total elimination of *C. jejuni* was not achieved. Due to the low infective dose of *C. jejuni* in humans (Black et al., 1988), the use of UV irradiation alone or in combination with activated oxygen cannot be recommended as the primary decontamination method for *C. jejuni* on broiler carcasses. The use of the methods in combination with other decontamination techniques and processing with good processing plant sanitation and hygiene might be more effective in reducing the *C. jejuni* counts on the broiler carcass surfaces than the use of these methods only.

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ARTICLE III

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"Wines as possible meat marinade ingredients possess antimicrobial potential against *Campylobacter*"

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Wines as possible meat marinade ingredients possess antimicrobial potential against *Campylobacter*

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ABSTRACT This research studied the survival of high (7 log cfu/mL) and low (3 log cfu/mL) inoculum levels of *Campylobacter* in white and red wines and in grape and tomato juices, which could function as potential antimicrobial marinade ingredients. For comparison, survival was also studied in a commercial poultry meat marinade. White and red wines were shown to have very high bactericidal effects against *Campylobacter*. High counts were rapidly inactivated to undetectable numbers within 15 min in white wine and within 1 h in red wine, and low counts within 15 min in white wine and within 30 min in red wine. By contrast, grape and tomato juices did not possess high bactericidal effects against *Campylobacter* because even low counts were occasionally detected after 48 h. The commercial marinade had rather high bactericidal effects against *Campylobacter*; the high counts were inactivated in most cases within 48 h, and all the low counts were inactivated within 3 h. When testing chicken meat inoculated with *Campylobacter* and subsequently submerged in white or red wine, the antibacterial activity of the wine was largely reduced. Wines lowered the *Campylobacter* load inoculated on chicken meat by approximately 1 log cfu/mL over 48 h. The results suggest that wines could be used as antimicrobial ingredients together with the addition of further antimicrobial agents in meat marinades to reduce the numbers of *Campylobacter* in naturally contaminated poultry products, thus lowering the risk of *Campylobacter* cross-contamination and transmission through food.

Key words: *Campylobacter*, survival, chicken meat, wine, juice

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INTRODUCTION

Campylobacter are the most common cause of foodborne bacterial gastroenteritis in humans worldwide (Samuel et al., 2004; European Food Safety Authority, 2010). The incidence of campylobacteriosis has increased steadily over the years, and it currently exceeds the number of cases of *Salmonella* infection (European Food Safety Authority, 2010). Important sources of *Campylobacter* infection are surface water, water from private wells, natural waters (via swimming), and unpasteurized milk (Park, 2002; Humphrey et al., 2007; Jacobs-Reitsma et al., 2008). However, the consumption and handling of improperly prepared poultry meat is considered one of the other major risk factors (Potter et al., 2003; Wingstrand et al., 2006; Jacobs-Reitsma et al., 2008).

A significant amount of poultry meat at the retail level is sold marinated in a wide range of flavors. Marinades are complex spiced, acidic water-oil emulsions typically containing salt, sugar, and sorbate, benzoate, or both. The high NaCl concentration, low pH, and addition of different spices to the marinades prevent the growth of spoilage bacteria, thus increasing the shelf life of meat products (Björkroth, 2005). Nevertheless, *Campylobacter* have been detected in different countries in both marinated and nonmarinated poultry products at the retail level, with a prevalence of between 10 and 100% and counts between <10 and 2.4 log cfu/g (Hänninen et al., 2000; National Veterinary and Food Research Institute, 2003; Kang et al., 2006; Scherer et al., 2006; Atanassova et al., 2007; Jacobs-Reitsma et al., 2008; Katzav et al., 2008; Pointon et al., 2008).

One potential approach to reducing the counts of *Campylobacter* that may be present in poultry meat could be to add substances with antimicrobial properties to the marinades used. Several studies have described the effect of the antimicrobial properties of wines on food pathogens such as *Bacillus* spp., *Escheri-*

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chia coli O157:H7, *Listeria* spp., *Salmonella* spp., and *Staphylococcus aureus* (Sugita-Konishi et al., 2001; Just and Daeschel, 2003; Møretrø and Daeschel, 2004; Fernandes et al., 2007; Waite and Daeschel, 2007; Hakovirta, 2008). The antimicrobial property of wine against *Campylobacter jejuni* was previously reported by Carneiro et al. (2008) and Birk and Knøchel (2009). The properties thought to contribute to the antimicrobial characteristics of wine are its low pH, presence of polyphenol compounds, high alcohol content, and high organic acid content (such as tartaric, acetic, lactic, malic, and citric acids; Just and Daeschel, 2003; Møretrø and Daeschel, 2004). In addition to wines, grape juice has been shown to have bactericidal effects against *Bacillus* spp. and *Listeria* spp., with the polymeric phenolic fractions being responsible for antilisterial effects (Rhodes et al., 2006; Hakovirta, 2008). Many different flavors are used in poultry meat marinades. In Finland, approximately 5% of poultry meat marinades contain tomato in powder, purée, or aroma form (depending on the manufacturer). In addition to their flavor properties, tomatoes have many beneficial nutritional and bioactive components (Du et al., 2008). However, only limited data are available on the antimicrobial effect of wines and juices against *Campylobacter*.

The objective of this research was to study the survival of both high (7 log cfu/mL) and low (3 log cfu/mL) inoculum levels of *Campylobacter* (3 *C. jejuni* strains and 1 *Campylobacter coli* strain) in white wine, red wine, grape juice, and tomato juice, which could function as potential antimicrobial marinade ingredients. For comparison, survival was also studied in a commercial poultry meat marinade. In addition, the reduction of *Campylobacter* (1 *C. jejuni* and 1 *C. coli* strain) by white and red wine inoculated onto chicken meat was studied.

MATERIALS AND METHODS

Bacterial Strains

Four *Campylobacter* strains were used in this study: a sequenced clinical human isolate *C. jejuni* NCTC 11168 strain (**RefCJ**; Health Protection Agency, London, UK); a *C. jejuni* strain (**RetCJ29**) and a *C. coli* strain (**RetCC27**), both isolated from honey-marinated retail poultry meat in a previous study by Katzav et al. (2008; RetCJ29 from Finnish poultry meat and RetCC27 from Brazilian poultry meat); and a *C. jejuni* strain (**SlaCJ26**) isolated at a Finnish slaughterhouse from turkey cecum content (laboratory collection). The RetCJ29, RetCC27, and SlaCJ26 were identified according to a modified method of the National Committee of Food Analysis (2007). The strains were maintained at −75°C in *Brucella* broth (02-042, Scharlau Chemie, Barcelona, Spain) containing 15% glycerol.

Liquid Type Description

The white wine (Sauvignon Blanc 2006, Gallo Family Vineyards, Modesto, CA) had an alcohol content of 13% and contained sulfites. The red wine (Cabernet Sauvignon 2006, Gallo Family Vineyards) had an alcohol content of 13.5% and also contained sulfites. The grape juice (grape nectar, Valio Oy, Valio, Finland) was a 72% grape nectar juice made from juice concentrates containing grape juice, added vitamin C, and water, with no preservatives. The tomato juice (Natur tomato juice drink, Oy Marli Ab, Turku, Finland) was a 100% tomato juice drink manufactured from juice concentrates containing tomato juice, glucose-fructose syrup, sugar, salt, and water, with no preservatives. According to the manufacturer, the commercial poultry meat marinade contained water, turnip, rapeseed oil, honey-apple wine vinegar, maltodextrin, glucose, spices, salt (1.0%), stabilizers (E451, E450, E452), yeast extract, flavor enhancer (E621), thickeners (E412, E415), aromas, preservatives (E211, E202), and an acidity regulator (E330). The wines and juices were purchased from local shops, and the marinade was provided by a local poultry processor. All the liquids were stored at 4°C before the experiments and were used for inoculation immediately after opening the container. As a control, the survival of *Campylobacter* was studied in brain heart infusion (**BHI**) broth (LAB49, LabM, Lancashire, UK), which was prepared according to the manufacturer's instructions.

The pH of each liquid type was measured before initiation of the studies by using a standard pH meter (MeterLAB, Radiometer Analytical, Copenhagen, Denmark). To check the sterility of the liquids before the tests, 100 μL of each liquid was spread on casein-peptone soybean meal-peptone agar (CASO, Merck, Darmstadt, Germany) with 5% bovine blood and was incubated aerobically at 37°C for 24 h.

Preparation of Bacterial Suspension

To prepare the bacterial suspension used for inoculations, the cells from the frozen stock culture were plated onto a *Campylobacter* blood-free selective medium [modified CCDA-Preston (**mCCDA**), CM0739, Oxoid, Hampshire, UK] containing CCDA selective supplement (SR0155, Oxoid) and incubated at 42°C for 48 h under microaerobic conditions obtained by CampyGen (CN0035, Oxoid). Between 1 and 3 single colonies from the growth were suspended into 5 mL of BHI broth (LabM) that had been prewarmed at 42°C, and the suspension was incubated at 42°C for 24 h under microaerobic conditions obtained as described above. After incubation, the optical density of the suspension at 600 nm was adjusted to 0.1, corresponding to approximately 8 log cfu/mL. The log colony-forming units per milliliter of bacterial suspension were counted by serial

10-fold dilution in BHI broth (LabM) and by preparing 2 spread plates (mCCDA, Oxoid) from the appropriate dilutions.

Preparation of Chicken Meat

Chicken breast fillets were purchased from a local retail outlet and immediately tested for the absence of *Campylobacter* spp. Testing was done according to method ISO 10272-1:2006 (International Organization for Standardization, 2006). Portions of approximately 10 g were cut antiseptically, put in separate stomacher bags (BA6041, Seward, Worthing, UK), and stored at 18°C until use. Only *Campylobacter*-negative meat samples were included in the study.

Inoculation

Inoculation of the Liquids. For the experiments on the liquids, all 4 *Campylobacter* strains (*C. jejuni* strain RefCJ, *C. jejuni* strains RetCJ29 and SlaCJ26, and *C. coli* strain RetCC27) were used. To inoculate the liquids with high bacterial counts, 1 mL of the original suspension, containing approximately 8 log cfu/mL, was suspended into 9 mL of the liquid type studied to give a bacterial concentration of approximately 7 log cfu/mL. To inoculate the liquids with low bacterial counts, 1 mL from a 10^{-4} dilution tube (used earlier for counting the original log colony-forming units per milliliter of suspension) containing approximately 4 log cfu/mL was suspended in 9 mL of the liquid type studied to give a bacterial concentration of approximately 3 log cfu/mL. The inoculated liquids were always vortexed immediately after inoculation and before plating.

Inoculation of the Chicken Meat. For the experiments on chicken meat, 2 *Campylobacter* strains, RefCJ and RetCC27, were used. Frozen chicken meat samples were thawed at ambient temperature for 60 min. For each test, a 10-g meat sample was used. To inoculate the meat samples with high bacterial counts, 100 μL of the original suspension, containing approximately 7 to 8 log cfu/mL, was applied to the separate meat pieces. To inoculate the liquids with low bacterial counts, 100 μL from the 10^{-3} dilution tube containing approximately 5 log cfu/mL was applied to the separate pieces of meat. Inoculated pieces of meat were kept at room temperature for 20 min to allow possible attachment and diffusion. To each meat sample, 10 mL of the corresponding liquid [white wine, red wine, or phosphate PBS (Oxoid)] was added.

Determination of Reductions in the Liquids and in Chicken Meat-Liquid Samples

The survival of all 4 *Campylobacter* strains in each liquid type was monitored at <1 min, 15 min, 30 min, 1 h, 3 h, 24 h, and 48 h after inoculation. The survival of the *Campylobacter* strains in each meat-liquid type was monitored at 10 min, 15 min, 30 min, 1 h, 3 h, 24 h, and 48 h after inoculation. The inoculated liquids and meat-liquid samples were kept at room temperature until the 3-h time point and were then stored at 4°C. The meat-liquid samples were homogenized in a laboratory blender (Stomacher 400 Circulator, Seward) before *Campylobacter* cell count. The reductions in the high and low inoculum levels were determined with serial 10-fold dilutions in BHI broth (LabM) and by preparing 2 spread plates (mCCDA, Oxoid) from each dilution. All plates were incubated at 42°C for 48 h under microaerobic conditions obtained as described above, and the colonies were identified by colony morphology and Gram staining. The results were counted and expressed as log colony-forming units per milliliter based on averages from the duplicate spread plates. In the liquids, the detection limit was 1 log cfu/mL, and in the chicken meat-liquid samples, it was 2 log cfu/mL. The tests were replicated twice for each liquid type and 3 times for each meat-liquid sample, and the results of the test replications were averaged.

Statistical Analysis

The data from each time point and the statistical differences between the main effects of bacterial strains and liquid types were all determined separately. Because the data were not normally distributed, significance was determined by a nonparametric Kruskal-Wallis test. Analyses were performed by means of the statistical package SPSS 16.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Survival of Campylobacter in White and Red Wines, Grape and Tomato Juices, and the Commercial Marinade

Table 1 presents the survival of high and low inoculum levels of the 4 different *Campylobacter* strains in white and red wine, grape and tomato juice, and the commercial marinade after different exposure times. Of all the liquids, white wine had the strongest antibacterial effect on all 4 *Campylobacter* strains. High counts of all strains were inactivated within 15 min, and the low inoculum levels of RetCC27 and RefCJ were inactivated to undetectable numbers within <1 min in white wine. In red wine, high counts of all *Campylobacter* strains were reduced to low counts within 30 min and were inactivated within 1 h. The grape and tomato juices were less bactericidal than the wines. In the grape and tomato juices, the high inoculum levels of SlaCJ26, RetCJ29, and RefCJ were still detected after 48 h of exposure. The low counts of strains RefCJ and RetCJ29 in grape juice and of strain RetCJ29 in tomato juice were still detected after 48 h of exposure. In the commercial marinade, the high counts of most of

the *Campylobacter* strains were inactivated within 48 h of exposure, and all the low counts were inactivated to undetectable numbers within 3 h.

Survival of Campylobacter in Meat-Wine Samples

Figure 1 shows the survival of high and low inoculum levels of *C. jejuni* (RefCJ) and *C. coli* (RetCC27) in the meat-wine samples compared with survival of these strains in the meat-PBS samples. Red wine (Figure 1A and 1C) and white wine (Figure 1B and 1D) moderately reduced the *Campylobacter* load on chicken meat over the sampling period, by approximately 1 log cfu/mL, regardless of the *Campylobacter* species inoculated or the type of wine used. Nonetheless, *Campylobacter* were still detectable in higher numbers after 48 h of exposure to white and red wine when inoculated on meat.

Survival of Campylobacter in BHI Broth and Sterility and pH Measurements of the Liquids

The initial pH was 3.20 ± 0.07 in white wine, 3.79 ± 0.05 in red wine, 3.62 ± 0.07 in grape juice, 4.11 ± 0.07 in tomato juice, 4.16 ± 0.03 in the commercial marinade, and 7.36 ± 0.03 in the BHI broth control solution. No changes were observed in counts of the *Campylobacter* strains studied in the BHI broth (LabM) control solution within 48 h at either of the inoculum levels (data not shown). The sterility of each liquid type was checked before the studies and no contaminants were found in any of the liquids, except in the case of the commercial marinade, in which diverse background flora were observed on the plates.

Statistical Analysis

The statistical differences in the effects of the different liquid types at each time point were examined using the data acquired from the *Campylobacter* strains with both high and low inoculum levels. Statistically significant differences ($P \leq 0.001$) were observed in the survival of *Campylobacter* strains in the different liquid types at every time point, except at the 48-h time point with low inoculum levels. No statistically significant differences ($P \geq 0.05$) were observed between the different *Campylobacter* strains in each of the liquid types studied with either of the inoculum levels. The observed differences in *Campylobacter* counts of meat-wine samples compared with meat-PBS samples were not statistically significant.

DISCUSSION

White and red wines had a very high bactericidal effect against all 4 *Campylobacter* strains studied, but they differed in their antimicrobial potential. In white wine, high counts of the strains were rapidly inactivated to undetectable numbers within 15 min and low counts of 2 strains were inactivated within <1 min. In red wine, high counts could not be detected after 1 h and low counts could not be detected after 30 min (Table 1). Carneiro et al. (2008) found that *C. jejuni* was inactivated within 30 s in red wine (initial counts, 6 to 7 log cfu/mL). Differences in inactivation rates may be due to differences in the strains and growth phases, cultivation media, and condition of and variations in the composition of the wine. Birk and Knøchel (2009) showed that *C. jejuni* survived for 15 min at 4°C in undiluted red wine, but when the marinating temperature was raised to 42°C, the bacterium was not detectable after 1 min. Sources of wine have been shown to differ in their potential to inactivate bacteria. Ganan et al. (2009) found, in contrast to our study, that red and rosé wines were more effective than white wine against *C. jejuni*. The combination of ethanol, phenolic compounds, certain organic acids, sulfur dioxide, and low pH (≤3.0) have been reported to be responsible for reducing the bacterial counts of various food-borne pathogens (Møretrø and Daeschel, 2004; Waite and Daeschel, 2007; Carneiro et al., 2008; Ganan et al., 2009; Birk et al., 2010). In the current study, grape and tomato juices did not reduce the *Campylobacter* counts as effectively as wines (Table 1). Previous studies have shown that both juices possess antimicrobial activity against different food pathogens such as *Listeria* spp., *Salmonella menston*, *E. coli*, *S. aureus*, *Yersinia enterocolitica*, and *Bacillus cereus*, with different survival times reported (Harding and Maidment, 1996; Eribo and Ashenafi, 2003; Rhodes et al., 2006; Hakovirta, 2008). Our findings are in agreement with those of Just and Daeschel (2003), who showed that bacteria survive longer in grape juice than in red wine. Because the 2 liquids in the present study had a similar pH value (3.79 and 3.62), it seems that in addition to the ethanol in wine, the type of acid and the specific composition of the liquid played a significant role in the survival of *Campylobacter*.

The commercial marinade inactivated most of the high *Campylobacter* counts within 48 h of exposure and all the low counts within 3 h of exposure (Table 1). This observed bactericidal effect of the marinade is concordant with the results of Perko-Mäkelä et al. (2000), who found, in a plain marinade (pH 4.5), that a mixture of 7 *C. jejuni* strains was not detectable after 48 h (initial counts, 5.4 log cfu/mL). However, *C. jejuni* survived for 7 to 10 d in marinated and nonmarinated chicken meat, depending on the level of inoculum used (between log 1.1 and log 5.3 cfu/mL; Perko-Mäkelä et al., 2000). Thus, marinating did not decrease the survival of *C. jejuni* in chicken meat stored at 4°C. In our study, the antimicrobial effect of wine on *Campylobacter* was also largely reduced when the *Campylobacter* inoculated on meat was exposed to wine. The *Campylobacter* load on chicken meat submerged in white or red wine was re-

Table 1. Counts[1] (log cfu/mL) of *Campylobacter* strains[2] after various exposure times to the liquids studied

		RefCJ		RetCJ29		RetCC27		SlaCJ26	
Liquid type	Exposure time	High	Low	High	Low	High	Low	High	Low
Red wine	0 min	7.2 ± 0.1	3.2 ± 0.1	7.0 ± 0.2	3.0 ± 0.2	7.4 ± 0.0	3.4 ± 0.0	7.1 ± 0.0	3.1 ± 0.0
	<1 min	6.8 ± 0.5	2.6 ± 0.1	6.2 ± 0.4	2.3 ± 0.5	6.4 ± 0.6	2.5 ± 0.3	6.2 ± 0.3	2.7 ± 0.3
	15 min	<1.0	<1.0	3.0 ± 4.3	1.0 ± 1.4	1.0 ± 1.4	<1.0	<1.0	<1.0
	30 min	<1.0	<1.0	1.4 ± 2.0	<1.0	<1.0	<1.0	<1.0	<1.0
	1 h	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
White wine	0 min	7.1 ± 0.3	3.1 ± 0.3	6.8 ± 0.2	2.8 ± 0.2	7.7 ± 0.0	3.7 ± 0.0	6.6 ± 0.4	2.6 ± 0.4
	<1 min	2.2 ± 3.1	<1.0	4.8 ± 1.6	1.1 ± 1.6	1.4 ± 1.9	<1.0	4.3 ± 2.9	1.2 ± 1.6
	15 min	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Grape juice	0 min	7.4 ± 0.1	3.4 ± 0.1	7.2 ± 0.0	3.2 ± 0.0	7.5 ± 0.3	3.5 ± 0.3	7.2 ± 0.1	3.2 ± 0.1
	24 h	6.4 ± 0.3	2.5 ± 0.1	6.5 ± 0.2	2.8 ± 0.8	1.2 ± 1.8	<1.0	6.1 ± 0.1	1.0 ± 1.4
	48 h	5.6 ± 0.0	1.0 ± 1.4	5.2 ± 0.6	1.3 ± 1.8	<1.0	<1.0	5.1 ± 0.3	<1.0
Tomato juice	0 min	7.2 ± 0.1	3.2 ± 0.1	7.1 ± 0.1	3.1 ± 0.1	7.6 ± 0.3	3.6 ± 0.3	7.2 ± 0.2	3.2 ± 0.2
	24 h	6.7 ± 0.4	2.0 ± 0.0	6.8 ± 0.3	2.5 ± 0.4	1.2 ± 1.8	<1.0	6.4 ± 0.1	1.5 ± 2.1
	48 h	5.9 ± 0.4	<1.0	6.2 ± 0.8	1.1 ± 1.6	<1.0	<1.0	4.4 ± 0.1	<1.0
Marinade	0 min	7.2 ± 0.0	3.2 ± 0.0	7.1 ± 0.0	3.1 ± 0.0	7.7 ± 0.0	3.7 ± 0.0	7.5 ± 0.0	3.5 ± 0.0
	1 h	6.7 ± 0.1	3.0 ± 0.3	6.8 ± 0.0	2.8 ± 0.1	7.3 ± 0.5	1.6 ± 2.2	6.8 ± 0.2	2.4 ± 0.6
	3 h	5.5 ± 0.3	<1.0	5.1 ± 0.5	<1.0	5.0 ± 1.4	<1.0	4.1 ± 0.4	<1.0
	24 h	3.7 ± 0.4	<1.0	2.0 ± 0.0	<1.0	<1.0	<1.0	<1.0	<1.0
	48 h	1.0 ± 1.4	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0

[1]Mean ± SD (n = 2). Detection limit was 1 log cfu/mL.

[2]The following *Campylobacter* strains were used: a sequenced clinical human isolate *Campylobacter jejuni* NCTC 11168 strain (RefCJ; Health Protection Agency, London, UK); a *C. jejuni* strain (RetCJ29) and a *Campylobacter coli* strain (RetCC27), both isolated from honey-marinated retail poultry meat in a previous study by Katzav et al. (2008; RetCJ29 from Finnish poultry meat and RetCC27 from Brazilian poultry meat); and a *C. jejuni* strain (SlaCJ26) isolated at a Finnish slaughterhouse from turkey cecum content (laboratory collection).

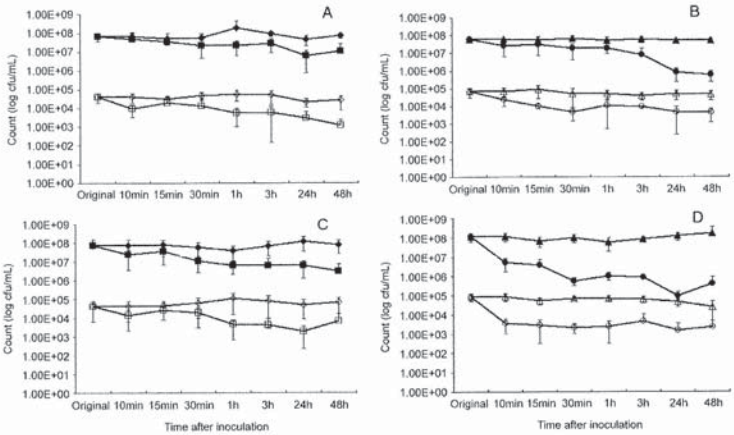

Figure 1. Survival of high and low counts of the *Campylobacter* strains RefCJ (a sequenced clinical human isolate *Campylobacter jejuni* NCTC 11168 strain, Health Protection Agency, London, UK; panels A and B) and RetCC27 (a *Campylobacter coli* strain isolated from honey-marinated retail Brazilian poultry meat in a previous study by Katzav et al., 2008; panels C and D) in red wine-marinated meat (panels A and C) and white wine-marinated meat (panels B and D) determined at different time points. RefCJ/RetCC27 counts in meat-PBS (◆, ◇ and ▲, △); RefCJ/RetCC27 counts in red wine-marinated meat (■, □); RefCJ/RetCC27 counts in white wine-marinated meat (●, ○). Error bars represent the SD.

duced by approximately 1 log cfu/mL after storage for 48 h at 4°C. With results corresponding to ours, Birk et al. (2007) found that red wine reduced the numbers of *C. jejuni* inoculated on chicken meat by only 0.5 log units (initial count, 7 log cfu/mL) after 3 d of storage at 4°C.

Based on results on the liquids only, the exposure of *Campylobacter* to white and red wines significantly reduced the number of viable cells. However, the antimicrobial activity of wines against *Campylobacter* was strongly reduced when their effects were tested on *Campylobacter* inoculated on chicken meat. The results suggest that wines could be used as antimicrobial ingredients in meat marinades together with further bactericidal agents, to reduce the numbers of *Campylobacter* in naturally contaminated poultry products. Björkroth (2005) has speculated that the buffering capacity of meat might neutralize the acidic components in marinades, leading to a decreased antimicrobial effect; however, the retail shelf life of refrigerated marinated chicken products is approximately 10 d (Perko-Mäkelä et al., 2000). Thus, even more effective results might be gained after the last time point used in this study (48 h).

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ARTICLE IV

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Short communication

Heat stress adaptation induces cross-protection against lethal acid stress conditions in *Arcobacter butzleri* but not in *Campylobacter jejuni*

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ABSTRACT

The ability of many bacteria to adapt to stressful conditions may later protect them against the same type of stress (specific adaptive response) or different types of stresses (multiple adaptive response, also termed cross-protection). *Arcobacter butzleri* and *Campylobacter jejuni* are close phylogenetic relatives that occur in many foods of animal origin and have been linked with human illness (mainly diarrhoea). In the present study, sublethal stress adaptation temperatures (48 °C and 10 °C) and mild and lethal acid conditions (pH 5.0 and pH 4.0) were determined for *A. butzleri* and *C. jejuni*. In addition, it was evaluated whether these sublethal stress adaptations cause specific adaptive responses or cross-protection against subsequent mild or lethal acid stresses in these bacteria. The studies were conducted in broth adjusted to the different conditions and the results were determined by the dilution series plating method. It was shown that heat stress adapted *A. butzleri* (incubated for 2 h at 48 °C) were significantly more resistant to subsequent lethal acid stress (pH 4.0) than non-adapted cells at the 1 h time-point ($p < 0.01$ in Wilcoxon rank sum test). No specific adaptive responses against the stresses in *A. butzleri* or *C. jejuni* and no cross-protection in *C. jejuni* were found. The ability of heat stressed *A. butzleri* to tolerate later lethal acid conditions should be taken into account when designing new food decontamination and processing strategies.

1. Introduction

Arcobacter (*A.*) spp. and *Campylobacter* (*C.*) spp. are close phylogenetic relatives that belong to the family *Campylobacteraceae* (Vandamme et al., 1991). Both genera have been linked with human illness. *Arcobacter* spp., especially *A. butzleri*, *A. skirrowii*, and *A. cryaerophilus*, have been associated with cases of bacteraemia and diarrhoea in humans (Collado and Figueras, 2011; Ho et al., 2006). *A. butzleri* was very recently defined as a potential zoonotic agent, a microbe of significant importance (Cardoen et al., 2009; Collado and Figueras, 2011), and considered for the first time as the etiological agent for traveller's diarrhoea (Jiang et al., 2010). *Campylobacter* spp., especially *C. jejuni* and *C. coli*, are the most frequently reported causes of bacterial gastroenteric infections worldwide (Park, 2002; Silva et al., 2011).

The most important potential transmission routes of *Arcobacter* spp. to humans are considered to be the consumption of contaminated food or water. *Arcobacter* spp. are frequently isolated from food products of animal origin, such as poultry (highest isolation rate), pork, beef and from various types of environmental waters (Collado et al., 2009; Collado and Figueras, 2011; Ho et al., 2006). Important sources of *Campylobacter* spp. infections are the consumption and handling of improperly prepared poultry meat, activities relating to recreational waters, contact with farm animals or pets and the drinking of unpasteurized milk (Humphrey et al., 2007; Silva et al., 2011).

Because *Arcobacter* spp. and *Campylobacter* spp. have been linked with human illness, efforts should be made to reduce their prevalence in foods. High temperatures, for example, are used for scalding during poultry slaughter (Van Driessche and Houf, 2008). Hilton et al. (2001) and D'Sa and Harrison (2005) have determined the decimal reduction times (*D*-values) for *A. butzleri* which range from 5.81 min at 50 °C to 0.4 min at 55 °C. *C. jejuni* is also easily inactivated by heat treatments (*D*-value 1 min at 55 °C; Jackson et al., 2009). These bacteria are also sensitive to low pH values and do not survive pH 4.0 (Cervenka, 2007; Jackson et al., 2009). However, the ability of many bacteria to adapt to stressful conditions may later protect them against the same type of stress or different types of stresses,

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phenomena known as specific adaptive response or as multiple adaptive response, also termed cross-protection (Xu et al., 2008). *C. jejuni*, for example, has been shown to tolerate acid stress more effectively after previous adaptation to mild acid stress conditions (Ma et al., 2009; Murphy et al., 2003). In addition, Klančnik et al. (2009) found that starved *C. jejuni* cells were able to withstand heat stress more effectively. Reid et al. (2008) reported that genes involved in heat shock response in *C. jejuni* were upregulated in response to acid stress, too. However, to the authors' knowledge, neither the ability of *A. butzleri* to adapt to stressful conditions, nor the protective effects of temperature stress adaptations on persistence of *C. jejuni* in acidic conditions has been studied previously. These bacteria are exposed to multiple stresses during their lifecycle in the food chain and are still able to survive. Thus, it is important to understand the effects of the stress conditions on their survival to control their presence in foods. Although *A. butzleri* and *C. jejuni* are close phylogenetic relatives, their survival under different conditions might differ and must therefore be studied separately. Both, *Arcobacter* spp. and *Campylobacter* spp., grow optimally under microaerobic conditions (3–10% O_2), but unlike most *Campylobacter* spp., *Arcobacter* spp. is also able to grow under aerobic conditions and below 30 °C (Kjeldgaard et al., 2009; Lehner et al., 2005; Snelling et al., 2006).

The objectives of this study were to determine the stress adaptation temperatures and pH conditions that cause a sublethal heat or acid stress, cold shock or lethal acid stress to the *A. butzleri* and *C. jejuni* strains studied. Furthermore, it was evaluated whether these sublethal stress adaptations cause specific adaptive responses or cross-protection against consecutive mild or lethal acid stresses in these bacteria.

2. Materials and methods

2.1. Bacterial strains and preparation of bacterial suspension

Two bacterial strains were used in this study: *A. butzleri* strain ATCC 49616 obtained from LGC Standards (Teddington, UK) and *C. jejuni* strain NCTC 11168 obtained from the National Collection of Type Cultures, Health Protection Agency, Centre for Infections (London, UK). Both strains are clinical human isolates. The strains were maintained at −80 °C in freezing broth containing distilled water supplemented with 0.5% (w/v) sodium chloride (106404, Merck KGaA, Darmstadt, Germany), 0.5% (w/v) meat extract (103979, Merck), 1% (w/v) peptone from casein (107213, Merck), 2% (w/v) D(+)-Glucose (108337, Merck), 25% (v/v) glycerol bidistilled 99.5% (24388.295, VWR International S.A.S., Fontenay-sous-Bois, France), and 20% (v/v) horse serum (BT-PG50, Labema Oy, Kerava, Finland).

To prepare the bacterial suspension used for inoculations, cells from the frozen stock culture were plated onto Mueller-Hinton agar with Sheep Blood (MHSB; PB5007A, Oxoid Ltd., Hampshire, UK) and incubated at 37 °C for 48–72 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Throughout the studies, the microaerobic atmospheres were generated using the CampyGen system (CN0035, Oxoid). One to five single colonies were suspended into 5 mL of pre-warmed (37 °C) Brain Heart Infusion (BHI) broth (CM1135, Oxoid) and the suspension was incubated at 37 °C for 24 h under microaerobic conditions. After incubation, the optical density of the suspension at 600 nm (OD_{600}) was adjusted to 0.1 using 37 °C BHI broth. This OD_{600} corresponds approximately to 10^8 CFU/mL.

2.2. Study conditions and bacterial inoculation

To determine sublethal stress adaptation conditions, survival of bacterial strains was studied individually at 48 °C (heat stress), at 10 °C (cold shock) and at pH 5.0 (mild acid stress). In addition, to determine the lethal acid stress conditions, their survival was studied at pH 4.0. Both acid stress studies were conducted at 37 °C. The different temperatures and pH values used in these determinations were chosen based on earlier survival studies conducted on *A. butzleri* and *C. jejuni* (Cervenka, 2007; Jackson et al., 2009; Kjeldgaard et al., 2009; Murphy et al., 2006; Van Driessche and Houf, 2008) and on own preliminary survival experiments conducted in the laboratory (data not shown).

For inoculation, 1 mL of the suspension containing about 10^8 CFU cells was suspended into 9 mL BHI broth (Oxoid) resulting in an original bacterial concentration of approximately 10^7 CFU/mL. Before inoculation with the bacterial suspension, BHI broth (Oxoid) tubes were adjusted to the different study temperatures (48 °C, 10 °C, or 37 °C). The pH of BHI broth (Oxoid) was adjusted to pH 5.0 or pH 4.0 with filter-sterilized (SLHA033SS, MILLEX-HA Filter Unit 0.45 μm, MILLIPORE, Carrigtwohill, Ireland) 15% (w/v) Tartaric acid (T-109, Sigma–Aldrich Co. Ltd., St. Louis, MO).

In the cross-protection study, cells were first inoculated as described above and adapted individually to heat stress (2 h at 48 °C), cold shock (24 h at 10 °C) or mild acid stress (4 h at pH 5.0). After adaptations, survival of the bacterial strains was studied individually under mild or lethal acid stress conditions by inoculating 1 mL of the adapted cells into 9 mL BHI broth with a pH of 5.0 or 4.0 (at 37 °C), resulting in an original bacterial concentration of about 10^6 CFU/mL.

The liquids in the study were always vortexed immediately after inoculation and incubated under microaerobic conditions at the temperatures studied.

2.3. Determination of reductions

Survival of the bacterial strains under each condition was monitored at time-points of 0 h, 1 h (at pH 4.0 only), 2 h, 4 h and 24 h after inoculation. The bacterial concentration of each suspension at the different time-points was determined via serial 10-fold dilutions in 9 mL BHI broth (Oxoid) and by inoculating two spread plates (MHSB, Oxoid) with 100 μl of bacterial suspension from each dilution. All plates were incubated at 37 °C for 48 h under microaerobic conditions. The results were counted and expressed as CFU/mL based on averages of the duplicate spread plates. The detection limit in the studies was 1 $\log_{10}$ CFU/mL.

2.4. Statistical analysis

In order to assure the reproducibility of the findings, the experiments were replicated at least four times for each study condition. More replicates were performed on the condition under which cross-protection was found likely, i.e., heat stress adapted *A. butzleri* (Table 1 and Fig. 1). Survival at pH 4.0 at 1 h for non-adapted *A. butzleri* was studied altogether 12 times and 16 times for heat stress adapted *A. butzleri*. At each time point, differences in the medians of viable bacteria counts under the two conditions were analysed using Wilcoxon rank sum test (R, Vienna, Austria). More precisely, the one-tailed version of the test was used to examine whether more bacteria that were viable resulted after stress adaptation than without adaptation.

3. Results

3.1. Determination of stress adaptation conditions

Table 1 shows the survival of *A. butzleri* and *C. jejuni* at 48 °C, 10 °C, pH 5.0 and pH 4.0. At 48 °C, counts of *A. butzleri* and *C. jejuni* decreased to undetectable numbers within 24 h. However, after 2 h

Table 1
Counts[a] (log CFU/mL) of *C. jejuni* and *A. butzleri* during exposure at 48 °C, 10 °C, pH 5.0 and pH 4.0 without adaptation and after heat or acid stress or cold shock adaptations. The bold values indicate the comparison where cross-protection was observed. Detection limit was 1 log CFU/mL.

Condition	Exposure time (h)	*C. jejuni*				*A. butzleri*			
		Non-adapted	Heat-adapted	Cold-adapted	Acid-adapted	Non-adapted	Heat-adapted	Cold-adapted	Acid-adapted
48 °C	0	7.6 ± 0.1	nd[b]	nd	nd	7.7 ± 0.2	nd	nd	nd
	2	7.3 ± 0.3	nd	nd	nd	6.7 ± 1.0	nd	nd	nd
	4	7.2 ± 0.4	nd	nd	nd	5.4 ± 1.2	nd	nd	nd
	24	<1.0	nd	nd	nd	<1.0	nd	nd	nd
10 °C	0	7.6 ± 0.2	nd	nd	nd	7.9 ± 0.2	nd	nd	nd
	2	7.6 ± 0.2	nd	nd	nd	7.4 ± 0.1	nd	nd	nd
	4	7.6 ± 0.2	nd	nd	nd	7.5 ± 0.1	nd	nd	nd
	24	7.5 ± 0.3	nd	nd	nd	7.0 ± 0.1	nd	nd	nd
pH 5.0	0	7.5 ± 0.3	6.6 ± 0.0	6.6 ± 0.2	nd	7.6 ± 0.1	6.4 ± 0.1	6.0 ± 0.1	nd
	2	7.4 ± 0.2	6.4 ± 0.1	6.4 ± 0.3	nd	7.7 ± 0.1	6.2 ± 0.1	5.6 ± 0.1	nd
	4	7.3 ± 0.3	6.3 ± 0.1	6.5 ± 0.2	nd	7.5 ± 0.2	6.1 ± 0.1	5.4 ± 0.1	nd
	24	7.1 ± 0.3	3.4 ± 2.3	4.6 ± 0.2	nd	5.6 ± 0.1	<1.0	<1.0	nd
pH 4.0	0	7.7 ± 0.1	6.5 ± 0.0	6.1 ± 0.3	6.4 ± 0.1	7.6 ± 0.2[c]	6.1 ± 0.2[d]	6.4 ± 0.4	6.4 ± 0.1
	1	7.5 ± 0.1	6.3 ± 0.2	5.7 ± 0.2	5.9 ± 0.9	**<1.0**[c, e]	**1.0 ± 1.3**[d, e]	<1.0	<1.0
	2	6.9 ± 0.1	5.1 ± 1.4	3.9 ± 0.8	2.9 ± 1.9	<1.0[c]	<1.0[d]	<1.0	<1.0
	4	3.7 ± 0.8	0.6 ± 1.2	<1.0	<1.0	<1.0[c]	<1.0[d]	<1.0	<1.0
	24	<1.0	<1.0	<1.0	<1.0	<1.0[c]	<1.0[d]	<1.0	<1.0

[a] Mean ($n = 4$) ± standard deviation.
[b] Not determined.
[c] Mean ($n = 12$) ± standard deviation.
[d] Mean ($n = 16$) ± standard deviation.
[e] $p < 0.01$.

at 48 °C, counts of both bacteria had decreased only moderately. A moderate decrease in the bacterial counts was also seen after 24 h incubation at 10 °C. Thus, sublethal heat stress and cold shock adaptations of the bacteria were determined to be conducted by incubating the cells for 2 h at 48 °C or for 24 h at 10 °C, respectively. The mild acid stress adaptations were chosen to be performed by incubating *A. butzleri* and *C. jejuni* cells for 4 h at pH 5.0, because after that their counts had moderately decreased. Incubation at pH 4.0 was chosen to represent the lethal acid stress condition, because under these conditions *A. butzleri* counts were inactivated to undetectable counts within 1 h and for *C. jejuni* within 24 h (Table 1).

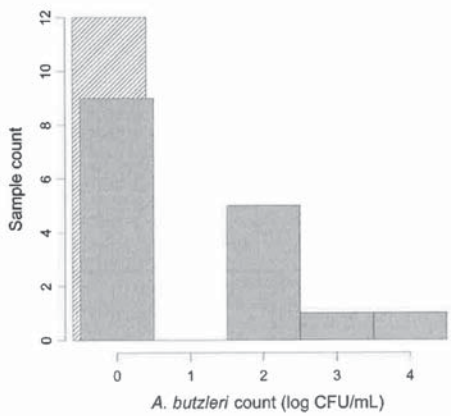

Fig. 1. Distributions of viable *A. butzleri* cell counts at pH 4.0 at the 1 h time-point without adaptation (column with stripes) and after heat stress adaptation (columns without stripes).

3.2. Cross-protection study

3.2.1. Survival of stress adapted bacteria at pH 5.0

Table 1 presents the survival of *A. butzleri* and *C. jejuni* at pH 5.0 after heat stress or cold shock adaptations and, for comparison, without adaptation. The data shows that the adaptations did not improve the survival of the bacteria at pH 5.0. This was also confirmed by the statistical analysis that did not find evidence for better survival after adaptation than without adaptation.

3.2.2. Survival of stress adapted bacteria at pH 4.0

The survival of *A. butzleri* and *C. jejuni* at pH 4.0 after heat or mild acid stress or cold shock adaptations and, for comparison, without adaptation is seen in Table 1. For *A. butzleri*, heat stress adaptation improved survival at the 1 h time-point at pH 4.0 (Table 1 and Fig. 1). The heat stress adapted *A. butzleri* cells were significantly ($p < 0.01$) more resistant to subsequent lethal acid stress than non-adapted cells at the 1 h time-point. In addition to Table 1 showing means and standard deviations of the data, the situation is depicted in more detail in Fig. 1. It can be seen that non-adapted *A. butzleri* cells did not survive at pH 4.0 in any of the study replications while viable heat adapted cells were counted up to 10^4 CFU/mL at the 1 h time-point. All non-adapted *A. butzleri* cells were inactivated to undetectable counts within 1 h at pH 4.0 (in 12 out of 12 study replications), whereas heat stress adapted *A. butzleri* cells were still detectable after 1 h at pH 4.0 in seven cases of the study replications ($n = 16$; Fig. 1). Under any other condition studied, the statistical test did not show evidence for cross-protection. At later time-points (2 h, 4 h and 24 h) at pH 4.0, no significant differences between the survival of heat stress adapted and non-adapted *A. butzleri* were observed. For *C. jejuni*, heat stress adaptation did not improve survival of the bacteria at pH 4.0, compared with non-adapted. For both bacteria, the mild acid stress or cold shock adaptations did not improve the survival of the bacteria at pH 4.0, compared with non-adapted. Like non-adapted *A. butzleri* cells, the mild acid stress or cold shock adapted cells were inactivated to undetectable counts within 1 h at pH 4.0. For *C. jejuni*, the cold- or acid adaptations actually decreased the survival times in pH 4.0, because the adapted cells were

inactivated to undetectable counts within 4 h (non-adapted within 24 h; Table 1).

4. Discussion

As far as we are aware, this is the first time cross-protection is reported for *A. butzleri*. In this study, heat stress adapted *A. butzleri* cells were shown to be more resistant to subsequent lethal acid stress than non-adapted cells at the 1 h time-point (Table 1 and Fig. 1). The adaptations might affect the survival of these pathogenic bacteria in food processing environments. Previously, specific adaptive responses and cross-protection against different stress conditions have been reported for *C. jejuni*, but to the best of the authors' knowledge, no comparative *Arcobacter* spp. studies have been published to date. Murphy et al. (2003) described the abilities of *C. jejuni* to survive better under lethal pH conditions after initial adaptation to mild acid stress or aerobic stress, or both. Ma et al. (2009) demonstrated that *C. jejuni* cells adapted to acid-, acid and aerobic-, or starvation conditions, were able to better withstand further acid challenges than the non-stressed cells. An adaptation to heat after prior heat shock has also been described in this organism (Mihaljevic et al., 2007). In addition, it has been shown that starved *C. jejuni* cells were able to withstand heat stress (Klančnik et al., 2009). Cross-protection at the gene expression levels was reported by Reid et al. (2008), where genes involved in heat shock response in *C. jejuni* were upregulated in response to acid stress, too. However, in this study the heat stress or cold shock adaptations of *A. butzleri* and *C. jejuni* did not improve their survival at pH 5.0, but actually lowered survival of the bacteria there. Mild acid stress or cold shock adaptations did not improve the survival of either bacteria at pH 4.0, but again actually decreased the survival times in some *C. jejuni* cases. For *C. jejuni*, the heat stress adaptation did not improve the survival of the bacteria at pH 4.0, when compared to non-adapted cells (Table 1). The use of bacterial strains always from the same growth conditions and growth phase might explain why specific adaptive responses or more cross-protection were not found in this study. Murphy et al. (2003), for example, showed that the induction of an adaptive tolerance response in *C. jejuni* was primarily dependent on the growth phase of the cells. In addition, the growth of *C. jejuni* in different media can lead to different adaptive responses (Murphy et al., 2005). It is also important to note, that these studies were conducted using bacterial collection strains which have been subjected to multiple subcultivations. The survival of wild type strains may be significantly different from these strains. Thus, additional work using more bacterial strains from different sources, growth conditions and growth phases is needed. Interestingly, the heat stress adapted *A. butzleri* cells were more resistant to subsequent lethal acid stress than non-adapted cells only at the 1 h time-point (Table 1 and Fig. 1). Ma et al. (2009) also reported that acid-, acid and aerobic-, or starvation adapted cells survived further acid stress more effectively than the non-adapted cells only in some *C. jejuni* strains used in the study, and that the stress-induced adaptive tolerance response in further acid stress was time dependent, i.e. detected only at certain time-points. In addition, different periods and conditions of adaptations were used here, compared to earlier studies. Furthermore, in pH studies, the survival of bacteria depends not only on the pH value, but also on the acid used, as some acids are more effective against *A. butzleri* even though the pH is the same (Cervenka, 2007).

At 10 °C and at 48 °C, the survival curves of *A. butzleri* and *C. jejuni* were quite similar (Table 1). A moderate decrease in the bacterial counts was seen after 24 h incubation at 10 °C. Comparative to this study, Phillips and Duggan (2002) observed a 3.02 $\log_{10}$ reduction in *A. butzleri* counts after 6.5 h of incubation at 10 °C, and a 2.0 $\log_{10}$ reduction after 4.5 h, but still survival of bacteria. Interestingly, Kjeldgaard et al. (2009) reported growth of *A. butzleri* ATCC 49616 at 10 °C in chicken meat juice medium (CMJ) and BHI without lag in CMJ but after $\geq$ 1 day in BHI broth. In our study, the *A. butzleri* counts could have also started to increase after the last time-point as described by Kjeldgaard et al. (2009). For *C. jejuni*, it was shown for various biological environments that survival at lower temperatures, such as at 4 °C, was much better than at 25 °C (Murphy et al., 2006). At 30 °C, its growth rate declines rapidly (Jackson et al., 2009). In the recent study, counts of *A. butzleri* and *C. jejuni* decreased to undetectable numbers within 24 h at 48 °C. However, after 2 h at 48 °C, the counts of both bacteria had decreased only moderately. Phillips and Duggan (2002) investigated the sensitivity of *A. butzleri* to 10 min treatments at 60 °C and 50 °C. Treatment at 60 °C, resulted in no colony forming units over the subsequent 24 h incubation at 30 °C, but after treatment at 50 °C, however, cells were detected after 24 h incubation at 30 °C (12.2% survival rate). For *C. jejuni*, growth at temperatures as high as 47 °C has been reported, but with an optimal growth temperature of 42 °C (Jackson et al., 2009). Thus, short treatments at temperatures around 48 °C, like those used in this study, seem to present sublethal heat stresses to *A. butzleri* and *C. jejuni*. At pH 4.0, on the other hand, *A. butzleri* cells were inactivated much faster than those of *C. jejuni*. In addition, after 24 h at pH 5.0, the log reduction in *A. butzleri* counts was higher than that seen in *C. jejuni* counts (Table 1). Based on these results, *C. jejuni* seems to be slightly more acid-tolerant, which corresponds to earlier studies. *Campylobacter* spp. grows optimally at pH 6.5–7.5 with minimum pH value at 4.9 and maximum at 9.0 (Chaveerach et al., 2003; Jackson et al., 2009), but *A. butzleri* grows between pH 5.0 to 8.5 (optimally between pH 6.0–8.0), and similarly to our study, with little if any growth below pH 5.0 (Cervenka, 2007; D'Sa and Harrison, 2005; Hilton et al., 2001).

In future, the cross-protective effect of heat stress adaptation on survival of *A. butzleri* in lethal acid conditions should be studied further at the gene expression levels, in order to elucidate molecular mechanisms behind this phenomenon reported.

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